Aspects of the molecular aetiologi of obesity and co-morbidities with a focus on impact of PPAR¿ gene variants and responses to dietary supplementation with conjugated linoleic fatty acids

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Aspects of the molecular aetiology of obesity and co-morbidities with a focus on impact of PPARγ gene variants and responses to dietary supplementation with conjugated linoleic fatty acids

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1) Larsen, T. M., L. H. Larsen, S. K. Torekov, J. Ek, E. Black, S. Toubro, A. Astrup, T. I. Sorensen, T. Hansen, and O. Pedersen, 2005, "Novel Variants in the Putative Peroxisome Proliferator-activated Receptor γ Promoter and Relationships with Obesity in Men," Obes. Res. 13, 953-958. (Larsen *et al.*, 2005b)

2) Larsen TM, Toubro A, Gudmundsen O, and Astrup A, 2005, "One year conjugated linoleic acid supplementation does not prevent weight or body fat regain.," Am J Clin Nutr **Submitted**. (Larsen TM *et al.*, 2005)

3) Larsen, T. M., S. Toubro, and A. Astrup, 2003, "PPARgamma agonists in the treatment of type II diabetes: is increased fatness commensurate with long-term efficacy?," Int. J. Obes. Relat Metab Disord. 27, 147-161.(Larsen *et al.*, 2003b)

4) Larsen, T. M., S. Toubro, and A. Astrup, 2003, "Efficacy and safety of dietary supplements containing CLA for the treatment of obesity: evidence from animal and human studies," J. Lipid Res. 44, 2234-2241.(Larsen *et al.*, 2003a)

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Content

Summary

Most westernized countries have experienced a major increase in the prevalence of obesity during the last decades. Obesity is one of the major underlying risk factors for the development of the metabolic syndrome, type-2 diabetes and cardiovascular disease. Much evidence suggests that common obesity is caused by several factors, including environmental and behavioural factors, as well as by a genetic susceptibility to an increasingly more obesogenic environment. Obviously, there is a need to provide further evidence about the contributing genetic factors to better formulate preventive and treatment strategies. There is also an acute need to identify safe inexpensive treatment options.

It is likely that the pathogenesis of obesity-associated diseases involves an abnormal fat distribution, usually identified and described as enlarged waist circumference and/or excessive visceral fat. Furthermore, several lines of evidence suggest that a more precise pathogenetic explanation is found in so-called ectopic fat deposition, i.e. that fat is deposited in non-fat body tissues, such as liver, skeletal muscle, heart and pancreas, which are not adapted to handle excessive amounts of fat. When chronically exposed to excessive amounts of fat, these organs become dysfunctional. Hence, there is evidence that obesity – at least when characterized as body mass index, is not *per se* the real underlying disease-causing factor. Thus, optimally, any treatment strategy should target the abnormal fat distribution, rather than obesity *per se*.
The transcription factor PPARγ is known to hold a central role in fat cell differentiation, and there are indications that modulation of PPARγ activity may inhibit ectopic fat deposition. It has been shown that agonistic activation of this transcription factor improves insulin sensitivity and regulation of blood glucose and possibly also the risk of cardiovascular disease. However, the effects of a more subtle modulation of PPARγ activity, either via variation in the gene encoding the protein or via partial activation or deactivation using specific agonists (also including various fatty acids) that can be taken as part of the diet, are not yet clear. In paper 3 and 4 and in the present thesis, aspects of the current understanding of the role of PPARγ in fat cell differentiation, as well as the potential of treating obesity with conjugated linoleic acids (CLA) are discussed.

Several studies in humans have documented that rare mutations in the *PPARγ* gene have devastating effects and causes lipodystrophy as well as an increased risk of type-2 diabetes and atherosclerosis. Also common variants have been identified in the *PPARγ* gene. Some studies have found these associated with an increased risk of obesity and type-2 diabetes, but the literature is inconsistent.
In paper 1, we studied whether these inconsistent findings could be explained by hitherto un-identified variants within the *PPARγ2* promoter region. Screening for variants within the promoter region, spanning from base pair -3371 to +43 in a group of 83 either severely obese or type-2 diabetic patients identified a total of 8 variants, of which the 7 (-792, -816C>T, -882, -1505G>A, -1881C>T, -1884T>A and -2604T>C) had not been reported before. We identified different degrees of linkage disequilibrium between these and the previously identified variants -1953A>G, Pro12Ala and 1431C>T. However, using data from clinical investigations performed on a subgroup of men recruited from the ADIGEN cohort we were unable to document any major association between these variants and adult BMI, early-onset obesity or change in BMI during adulthood neither by associations with adult waist, fat mass, lean tissue mass or body fat percentage. This suggests that none of these variants contribute substantially to the pathogenesis of common obesity in this population.
Although we cannot clearly refute that common variants within the *PPARγ* locus may contribute to obesity and/or type-2 diabetes, our data suggest that such effects are relatively small or that they may depend on interaction with other – yet unidentified – genetic or environmental factors. The success of future association studies of common *PPARγ* variants will most likely depend on the availability of appropriate data, i.e. an optimal study design, which preferably should include large sample sizes with thorough and detailed phenotypic characterization (preferably including fat distribution using Magnetic Resonance or CT-scanning techniques) and descriptions of several environmental and

behavioural factors. Also, taking possible effects of various haplotypes, gene-gene and gene-environment effects into account would probably further increase success rate.

An accumulating number of studies have found that that specific conjugated linoleic acids (CLA), particularly the trans10, cis12-isomer have unique PPARγ receptor down-regulating properties, that are associated with inhibition of adipocyte differentiation and inhibition of fat tissue gain when studied *in vivo* in animals and possibly also in humans. In the paper 4, we set out to evaluate if a commercially available CLA dietary supplement would affect 1-year regain of body weight or body composition after a major initial weight loss, and whether treatment with CLA induced any adverse effects. Overall, we found no evidence that CLA did induce any changes impact on in body weight, body composition or fasting levels of blood glucose or insulin. However, we did find a marginally increased blood leukocyte count, which may indicate enhancement of inflammatory processes by CLA treatment. CLA-isomers are likely the most well-studied single fatty acid isomers, and the pertinent literature suggests that at least some of the specific CLA-isomers may have biological characteristics resembling other trans fatty acids, with which they are structurally related. Some of these characteristics suggest that, although they may possess anti-obesity properties, they do also seem to have modest adverse effects, some of which may increase insulin resistance and risk of cardiovascular disease.
In conclusion, CLA does not seem to be a promising agent for treatment of obesity, and the safety profile is not yet defined. However, the biological system with PPARγ as a central regulator of lipid metabolism and obesity may be a promising target for other novel drugs and bio-active food ingredients.

Dansk sammendrag

De fleste vestlige lande har oplevet en voldsom stigning i prævalensen af fedme i løbet af de seneste årtier. Fedme er en af de væsentligste underliggende risikofaktorer for udvikling af det metaboliske syndrom, type 2 diabetes og hjerte-kar sygdom. Det er veldokumenteret, at såkaldt simpel fedme opstår som et samspil mellem forskellige miljø- og adfærdsmæssige faktorer og en arvelig disposition som øger følsomheden overfor et stærkt fedmedisponerende miljø. Der er et åbenlyst behov for at få en bedre forståelse for de genetisk prædisponerende faktorer for dermed bedre at kunne tilrettelægge forebyggelses- og behandlingsmuligheder. Desuden er der også et akut behov for at finde nye effektive, sikre og billige behandlingsmetoder af fedme.

Patogenesen bag fedme-associerede sygdomme involverer en abnormal fedtfordeling, normalt identificeret og karakteriseret som en forøget talje-omkreds, og især øget visceral fedme. Ydermere er der dokumentation for at en mere præcis patogenetisk forklaring skal søges i den såkaldte ektopiske fedtaflejring, dvs. en fedt-ophobning som sker i væv og organer udenfor de almindelige fedtdepoter. Disse væv er typisk lever, muskler, hjerte og bugspytkirtel – væv som normalt ikke er udviklet til at deponere fedt. Når disse væv kronisk udsættes for denne overflod af fedt tager de skade. Således anses fedme i sig selv ikke for at være den egentlige sygdomsfremkaldende faktor. Optimalt set, så bør derfor enhver behandlingsstrategi målrettes mod denne abnormale fedtfordeling i stedet for mod kroppens totale fedtmasse

Transkriptionsfaktoren PPARγ har en central rolle i fedtcelle differentieringen, og en modulering af PPARγ aktiviteten kan hæmme den ektopiske fedtaflejring. Således er det vist at agonistisk aktivering af denne transkriptionsfaktor kan øge insulin følsomheden, således at blodglukose falder og muligvis også sænker risikoen for hjerte-kar sygdom. Effekten af en moderat modulering af PPARγ aktiviteten er til gengæld uklar, dette gælder hvad enten det drejer sig om betydningen af genetisk variation i PPARγ genet, eller om det induceres via en partiel aktivering eller hæmning ved hjælp af specifikke agonister eller antagonister (herunder også specifikke fedtsyrer som kan indtages som en del af kosten).
I artikel 3 og 4 og i denne phd-afhandling gennemgås og diskuteres den nuværende forståelse af PPARγ´s rolle i fedtcelle udviklingen samt de potentielle muligheder der er for at behandle fedme ved hjælp af konjugerede fedtsyrer (CLA) som sandsynligvis virker på PPARγ.

Flere humane studier har fundet at sjældne mutationer i *PPARγ* kan medføre udvikling af lipodystrofi samt give øget risiko for udvikling af type 2 diabetes og aterosklerose. Også mere hyppigt optrædende varianter er identificeret i *PPARγ.* Nogle studier har fundet, at disse almindelige varianter også kan give en øget risiko for udvikling af fedme og type 2 diabetes, men litteraturen er modstridende. I artikel nummer 1 har vi undersøgt om disse modstridende fund kunne forklares af effekten af hidtil ukendte varianter i *PPARγ2* promotor regionen. Denne screening for varianter i promotor regionen, spændende fra basepar nummer -3371 til +43 i en gruppe af 83 enten svært fede eller type 2 diabetes patienter identificerede 8 varianter, af hvilke de 7 (-792, -816C>T, -882, -1505G>A, -1881C>T, -1884T>A og -2604T>C) ikke var rapporteret tidligere. Vi fandt forskellig grad af genetisk kobling mellem disse og de tidligere identificerede varianter -1953A>G, Pro12Ala and 1431C>T.
Ud fra kliniske undersøgelser af en gruppe af mænd rekrutteret fra ADIGEN kohorten fandt vi dog ingen sammenhæng mellem de fundne genetiske varianter og BMI i voksenalderen, tidlig opstået fedme eller på ændring i BMI i voksenalderen. Vi fandt heller ingen sammenhæng i forhold til talje omkreds, fedtmasse, mager legemsmasse eller kropsfedtprocent i denne gruppe af mænd. Dette peger således på at ingen af disse varianter bidrager væsentligt til patogenesen bag almindelig fedme i denne population. Selvom vi ikke helt kan afvise at hyppige varianter i *PPARγ* regionen kan influere på risikoen for fedme og/eller type 2 diabetes, så viser disse undersøgelser at sådanne effekter under alle omstændigheder er ret begrænsede, eller at effekten afhænger af en interaktion med andre – endnu uidentificerede – genetiske eller miljømæssige faktorer. Succesen af fremtidige genetiske

associationsstudier af hyppige *PPARγ* varianter afhænger i høj grad af kvaliteten af de tilgængelige data, dvs. at et optimalt studiedesign helst skal inkludere et stort populationsmateriale med en grundig og detaljeret fænotypisk karakteristik (optimalt set også en bestemmelse af kropsfedtfordeling vurderet ved hjælp af magnetisk resonans eller CT-scanning) samt en beskrivelse af en vifte af miljø- og adfærdsmæssige faktorer. Yderligere inddragelse af haplotype-analyser, samt af gen-gen og gen-miljø effekter kan formodentlig øge succesraten yderligere.

Studier i en række dyremodeller og enkelte humane studier har fundet at specifikke konjugerede fedtsyrer (CLA), især trans10,cis12-isomeren, har unikke PPARγ receptor nedregulerende effekter, som er påvist at hæmme udviklingen af fedtceller og fedtvæv. I artikel 4 har vi undersøgt om et kommercielt tilgængeligt CLA kosttilskud kan påvirke kropsvægten eller fedtmassen hvis det gives i op til 1 år efter et diætinduceret større vægttab. Vi har desuden undersøgt eventuelle bivirkninger af CLA.
Overordnet fandt vi at CLA ikke gav ændringer i kropsvægt, kropssammensætning eller påvirkede fasteniveauet af glukose eller insulin i blodet, sammenlignet med placebo. Dog fandt vi at CLA medfører en lille øgning af leukocytter i blodet, et fund som indikerer en øget inflammation. CLA-isomerer er sandsynligvis de mest velstuderede enkelt-fedtsyre-isomerer, og den tilgængelige litteratur godtgør at i hvert fald nogle af disse specifikke CLA-isomerer har biologiske egenskaber som minder om andre transfedtsyrer, med hvilke de også er strukturelt relateret til. Selvom det tyder på at CLA-isomerer kan besidde anti-fedme egenskaber, så ser de også ud til at inducerede negative effekter, herunder en øgning af insulin resistens og en mulig forøgelse af risikoen for hjerte-kar sygdom.

Samlet set, så ser CLA ikke ud til at være en lovende behandling af fedme, og sikkerhedsaspektet er tillige endnu ikke fuldt ud undersøgt. Det er til gengæld klart at det biologiske system hvori PPARγ indgår, er en nøglefaktor i reguleringen af fedtstofskiftet og fedmeudviklingen og at det derfor kan være et lovende angrebspunkt for nye typer af medicin og bioaktive fødevareingredienser.

Preface

The papers presented in this thesis have been prepared in the years 2002 to 2005. The 1-year study of CLA-supplementation (paper 2) was initiated in January 2002 at the Department of Human Nutrition, The Royal Veterinary- and Agricultural University (RVAU), Frederiksberg, Denmark and at Hvidovre Hospital, Hvidovre, Denmark. The study was carried out under supervision of Associate Professor, MD, Søren Toubro and Professor, MD, dr.med.sci.Arne Astrup. The study was terminated at around May 2003.

The genetic analyses of PPARγ2 (paper 1) was carried out at Steno Diabetes Center, Gentofte, Denmark. The practical genetic work was carried out in the period from January to July 2003 under careful supervision of Msc. Lesli Larsen, PhD Jakob Ek and Professor, MD, Oluf Borbye Pedersen.

The review papers (paper 3 and 4) were prepared during 2003 and 2004, strongly encouraged by Arne Astrup and Søren Toubro, who also contributed to the papers.

Part of the work and the thesis is supported by a PhD Scholarship for the period July 2002 - June 2005. In addition, the CLA study was financed by Natural A/S, Norway. The PPARγ study was also supported by The Danish Diabetes Association, the Danish National Research Foundation and the Danish Heart Foundation which financially supported these studies at Steno Diabetes Center.

Hence, a large number of people have contributed to the completion of the papers in this thesis.
At Steno Diabetes Center I am particularly grateful to Oluf Borbye Pedersen: Thank you for providing me the opportunity to do the genetic studies at your department, and for your firm, consistent and always helpful and critical advice during the practical studies at the department and the subsequent writing. Also, I particularly want to thank Lesli Larsen for all her help and support. Finally a warm thanks to all the people in the group of Oluf Borbye Pedersen who made my stay at Steno Diabetes Center a pleasant and invaluable experience.

At the Department of Human Nutrition at RVAU, I would foremost like to thank my supervisor Arne Astrup. You have has been an unsurpassed inspirator, always being enthustiastic, encouraging and supportive. Also a warm thank to Søren Toubro for your always open door and welcoming attitude, your willingness to help and discuss pending issues, and for your always good (but sometimes black) humour. I would like to thank Benjamin Buemann, with whom I shared office during approx. 2 years. Thank you for always having the time and interest to discuss scientific issues and for being an excellent travelling partner at our trips to various scientific meetings.

I gratefully acknowledge the expert help from the technical and administrative staff at the Department of Human Nutrition and Hvidovre Hospital, without which these studies would not have been possible. Therefore, for excellent laboratory and technical help, I thank Kirsten Bryde Rasmussen, Inge Timmermann, Helle Reinikka Christensen and Martin Kreutzer; for excellent dietetic help I thank Ulla Pedersen, Annette Vedelspang, Kit Henningsen and Pia Gronemann. Finally, for secretarial or administrative help, I thank Heidi Jensen, Trine Stougaard Stasiak, Maj-britt Petersen and Maybritt Rydahl.

As it is no secret that other studies, particularly the MUFObes study have also taken some of my working time during the period from summer 2003 until now, and because this work is not described in this thesis, I would also like to thank the substantial number of people (not mentioned, not forgotten!) who have helped me – and us - during the substantial collaborative work carried out within the MUFObes study. This help I hope to be able to acknowledge further in the future.

In general, I would also like to thank the staff at the Department of Human Nutrition; i.e. Kitchen Staff, Laboratory and other Technical staff, Dietician Staff and the very long list of academic personal with whom I have shared time and fellowship. In particular I will thank my close colleague and current room mate Anette Due for all her help, for fruitful discussions and for being very patient and tolerant with me. Also, I sincerely thank the help of Camilla Verdich for her constructive critique of my work, including this thesis. I will thank my friends and my family for always being supportive, regardless if they might not fully understand what my work actually meant as such, and particularly what it meant to me. Fortunately, I have found substantial meaning of studying the implications of healthy eating, avoiding being obese and of sustaining a healthy lifestyle together with the possible

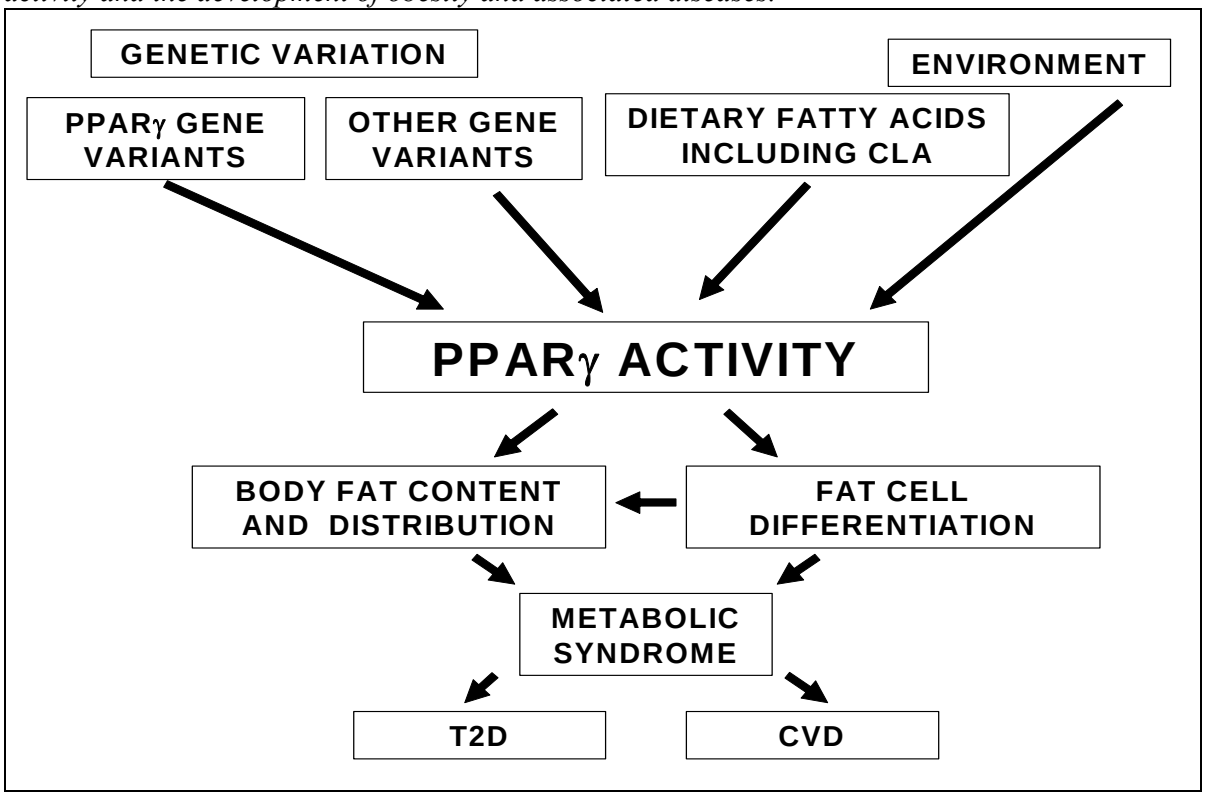
implications that genetic predisposition to diseases may have. The recent death of my father (deceased April 5, 2005), despite being a very sad event, has only further increased my interest into the studies of lifestyle diseases. Further appreciating the substantial knowledge that we already have about the significance of healthy living and healthy choices, and the importance of taking individual responsibility for our life, I believe will increase the quality of life (and life length) for most people.

September 2005
Thomas Meinert Larsen

1 Introduction

The aim of the present thesis is to make a thorough evaluation and perspectivation of the results presented in the two original papers (Larsen TM *et al.*, 2005a;Larsen TM *et al.*, 2005b). In particular to discuss the key findings in these papers in the context of the data currently available in the relevant literature, covering issues as diverse as fat cell differentiation, molecular-genetic studies, animal models, human epidemiological studies and human intervention studies. In order to appropriately cover these diverse areas, the discussion and interpretation of the 2 papers are put into the perspective of the two review papers (Larsen *et al.*, 2003a;Larsen *et al.*, 2003b) and include whatever pertinent data that have been published before and after these reviews. Hence, the findings already described in these reviews will not be referenced in all details, and instead the emphasis is put on other or more recent literature. It is emphasized that this thesis has obesity, the metabolic syndrome (MS), including risk of type 2 diabetes (T2D) and cardiovascular disease (CVD) as its primary areas, although variation within the *PPARγ* gene and supplementation of CLA may have significant biological effects on other diseases not particularly related to the pathogenesis of obesity, MS, T2D or CVD. The physiology of the PPARγ system and the metabolic effects of CLA-supplementation may at first sight appear to be two very different research areas. However, as it will hopefully appear in this thesis, and as illustrated in figure 1, an emerging amount of data supports the notion that the PPARγ system and dietary fatty acids, including CLA, do actually interact in multiple ways, affecting human metabolism and potentially also human disease. I hope that further exploration of these complex interactions, along with other interacting factors, may provide further evidence, which can be used to pave the way for more effective and more individualized prevention and treatment of obesity and to prevent obesity-related diseases.

Figure 1: The complex interaction between genetic variation and dietary fatty acids on PPARγ activity and the development of obesity and associated diseases.



Genetic variation within the PPAR*γ* gene together with environmental effects which also includes dietary fatty acids (i.e. also CLA) may regulate PPAR*γ* activity, particularly in fat cells. The effects on fat cells may influence the distribution of subcutaneous and visceral fat in the body, ultimately affecting the risk of developing the metabolic syndrome, type 2 diabetes and cardiovascular disease.

1.1 Obesity and associated diseases

Body weight relative to body height has been shown to increase the risk of various diseases, such as the MS,CVD and T2D, which are in focus in this thesis, but also certain forms of cancer (WHO, 2000). The WHO (WHO, 2000) has made classifications of BMI (kg/m^2) to categorize people according to increased risk of developing diseases (Figure 2).

Figure 2: Classification of overweight in adults according to BMI

Classification of overweight in adults according to BMI[a]

Classification	BMI (kg/m^2)	Risk of comorbidities
Underweight	<18.5	Low (but risk of other clinical problems increased)
Normal range	18.5–24.9	Average
Overweight	⩾25.0	
Pre-obese	25.0–29.9	Increased
Obese class I	30.0–34.9	Moderate
Obese class II	35.0–39.9	Severe
Obese class III	⩾40.0	Very severe

[a] These BMI values are age-independent and the same for both sexes. However, BMI may not correspond to the same degree of fatness in different populations due, in part, to differences in body proportions. The table shows a simplistic relationship between BMI and the risk of comorbidity, which can be affected by a range of factors, including the nature and the risk of comorbidity, which can be affected by a range of factors, including the nature of the diet, ethnic group and activity level. The risks associated with increasing BMI are continuous and graded and begin at a BMI below 25. The interpretation of BMI gradings in relation to risk may differ for different populations. Both BMI and a measure of fat distribution (waist circumference or waist : hip ratio (WHR)) are important in calculating the risk of obesity comorbidities.

Source: reference *26*.

Adapted from (WHO, 2000)

The pathophysiology of obesity is complex and so is also the exact calculation of relative risk of developing obesity-related co-morbidities. It can be argued that the BMI index is a rather inaccurate measure of obesity degree, and that it is too inaccurate to use it to assess effects on risk of disease. Though this may be true for the individual (as it will be discussed thoroughly in the sections below), epidemiological studies strongly suggest that being overweight (assessed via BMI) is an important predictor of all-cause mortality. This risk is independent but additive from the risk of being physically inactive (Hu *et al.*, 2004). Unfortunately, the prevalence of obesity is reaching epidemic proportions (International Obesity TaskForce & European Association for the Study of Obesity, 2002;Hedley *et al.*, 2004) and thus concerns for future increased medical costs and a potential decline in life expectancy have arisen (Olshansky *et al.*, 2005)

1.1.1 The metabolic syndrome

The excessive risk of disease associated with the obese state *per se* is controversial. Accumulating evidence suggest that obesity is merely the underlying cause of emerging risk factors. It has been known for decades that obesity is often associated with several risk factors for the development of T2D and CVD. Suggestions of a state of insulin resistance as a common associate to the development of diabetes was published as early as 1939, and the concept of syndrome X, a constellation of risk factors associated to the development of CVD was described by Gerald M Reaven in 1988 (Reaven GM, 2005). Subsequently this pattern of risk factors associated with the development of T2D and CVD has also been designated the insulin resistance syndrome or MS, and the American Heart Association, The US National Heart, Lund and Blood Institute and the American Diabetes Association now recommend using the wording “Metabolic Syndrome” (Expert Panel, 2001;Grundy *et al.*, 2004). Today, there are several – and slightly different – definitions of MS that are adapted by different organizations such as the American Diabetes Association (Expert Panel, 2001), WHO

(Alberti and Zimmet, 1998), and the International Diabetes Federation (www.idf.org, 2005). In addition, the American Association of Clinical Endocrinologists and the European Group for the study of Insulin Resistance 2002 (EGIR) have proposed alternative but similar clinical criteria for their description of the insulin resistance syndrome (Balkau *et al.*, 2002;Einhorn *et al.*, 2003). According to the National Health and Nutrition Examination Survey (NHANES) 1999-2000, the prevalence of MS, as defined by the National Cholesterol Education Program/Adult Treatment Panel III (NCEP/ATPIII) among US adult is approximately 27 % (Ford *et al.*, 2004). For comparison, in Denmark the prevalence of MS in women is around 15 % in 1999 as defined by the NCEP/ATPIII criteria (Lauenborg *et al.*, 2005). Unfortunately, these numbers are most likely on the rise in most westernized countries.

1.1.2 Type-2 diabetes

Similar to the metabolic syndrome, the use of diagnose and classifications of T2D are also different; WHO (Alberti and Zimmet, 1998) and ADA (American Diabetes Association, 2005) although they are nearly identical. In 2001, the prevalence of T2D in US adults was 7.9 % (Mokdad *et al.*, 2003), whereas it is estimated that 100.000-150.000 people in Denmark have diagnosed T2D, corresponding to 2-2,5 % of the adult (>20 years) population (Sundhedsstyrelsen, 2003). In addition, it is anticipated that a quite substantial number of persons have undiagnosed T2D. Obesity, and particularly indices of abdominal obesity, is one of the major underlying risk factors for the development of T2D, and the waist circumference seems the best predictor of T2D (or at least as good a predictor) compared to other anthropometric indexes of central obesity (Guerrero-Romero and Rodriguez-Moran, 2003;Mamtani and Kulkarni, 2005). A recent study in 909 korean women with a history of gestational diabetes found an odds ratio of 5.8 to develop T2D between patients within the upper and lower quartile of waist circumference (Cho *et al.*, 2005). Also, a study in swedish men found an 16.6 times higher risk of developing T2D in the group within the upper 5% distribution of waist/hip ratio when compared to the group within the lowest quintile (Ohlson *et al.*, 1985), the association being stronger than for BMI only. For a general description of important risk factors (including family history and diet) for development of T2D, the reader is advised to one of the several reviews and statements on this topic (Sheard *et al.*, 2004;Mann *et al.*, 2004;Klein *et al.*, 2004b). Finally, and of major importance, a study suggest that having a diagnosis of T2D reduces life expectancy with 7 years in men and 7.5 years in women, primarily as a result of increased risk of CVD (Morgan *et al.*, 2000).

1.1.3 Cardiovascular disease

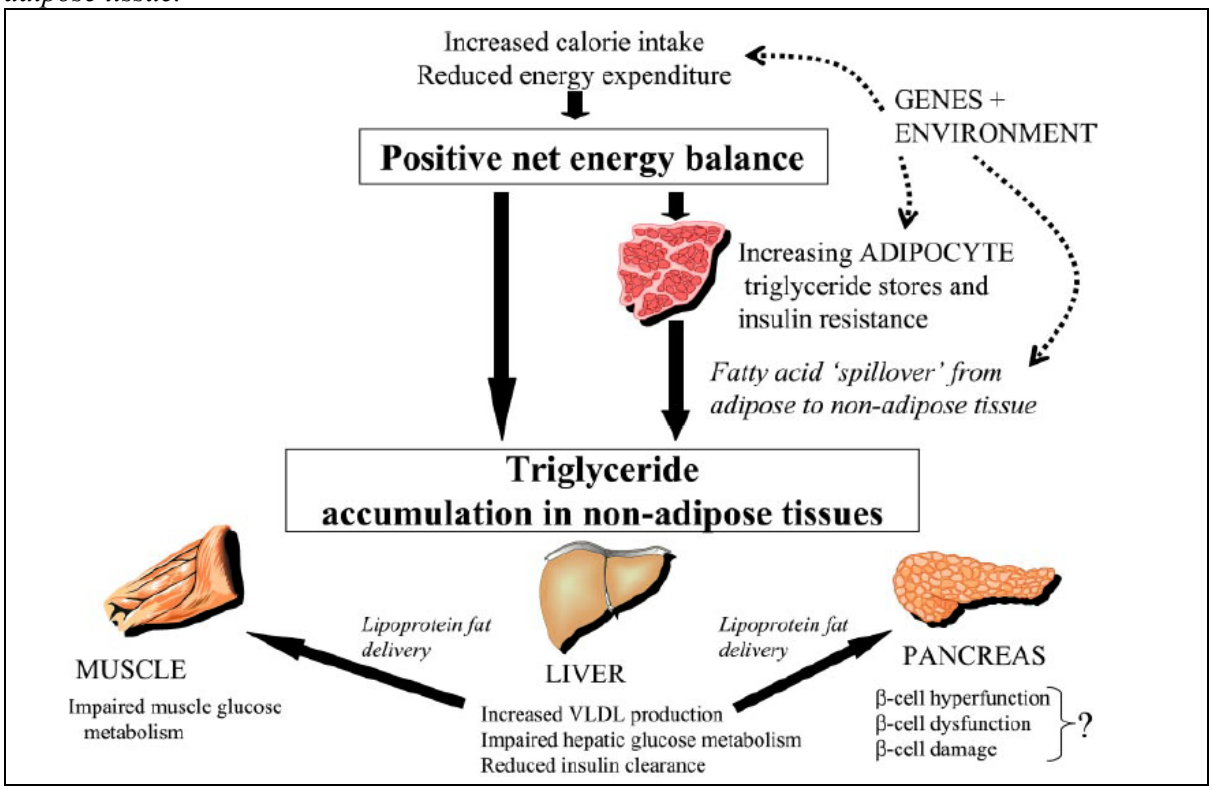
It is difficult to obtain a precise definition of atherosclerotic CVD. In this thesis, the description will include peripheral atherosclerosis, coronary ischemic disease and stroke although it is quite obvious that the underlying pathophysiological mechanisms may be somewhat different for these different conditions. The prevalence of peripheral arterial disease in the US adults aged >40 y, estimated by an ankle-brachial index >0.90 in the leg (an important marker of sub-clinical coronary heart disease) is approx. 4.3 % in the 1999-2000 National Health and Nutrition Examination Survey (Selvin and Erlinger, 2004). As discussed above, the risk factors known to cluster within MS are highly associated to the development of ischemic CVD, and particularly the diagnosis of T2D is a major risk factor for future CVD. It is generally agreed, that obesity *per se* does not affect the risk of CVD as much as for T2D, but obesity still increases the risk by a factor of approx. 2 (Kenchaiah *et al.*, 2002) (Nanchahal *et al.*, 2005) (Expert Panel, 2001).

1.2 Abnormal fat distribution – implications for adverse effects of fatness

Epidemiological studies support the notion that central adiposity (e.g. measurement of waist circumference) is a better predictor than BMI of the development of T2D in humans (Ohlson *et al.*, 1985) and also a better predictor of all-cause mortality (Bigaard *et al.*, 2004).
A recent study also suggest that body weight history, i.e. the time point for weight gain, may have substantial influence on subsequent risk of T2D, as late-onset obesity has been shown to be a better predictor of development of T2D than early onset obesity (Black *et al.*, 2005). This finding could be explained by an increased ratio of abdominal weight gain/total weight gain after the age of ~30 y compared to age <30 years. Upper-body obesity has been shown to be a significant risk factor of impaired glucose tolerance, whereas lower-body fat (adjusted for total body fat) seems to protect against impaired glucose tolerance (Buemann *et al.*, 2005) and against risk factors associated with CVD (Buemann B *et al.*, 2005).

Following the substantial evidence, that fat distribution (lower- or upper-body obesity) is a better predictor of disease than just obesity as assessed by BMI, total body fat, or body fat percentage (which do usually not give any information about the fat distribution). A substantial number of studies have indicated that particularly the upper-body obesity phenotype is associated with fat deposition in a variety of tissues not designed for storage of fat, such as liver, skeletal muscle, heart and pancreas. Several expressions have been proposed for the description of this condition, such as 'failure of adipocyte differentiation' (Danforth E Jr, 2000), 'ectopic lipids' (Unger, 2003), 'ectopic fat distribution' (Ravussin and Smith, 2002), 'disordered fat storage' (Lewis *et al.*, 2002) 'lipotoxicity' (Schaffer, 2003), and 'adipocyte insensitivity syndrome' (Erol, 2005).

Figure 3: Development of lipotoxicity when positive energy balance exceeds the buffering capacity of adipose tissue.



An increased calorie intake and a reduced energy expenditure, whether being a result of environmental and/or genetic effects, leads to a positive energy balance. The energy surplus will usually be deposited in adipose tissue. However, in situations of chronic positive energy balance, the adipose tissue cannot sufficiently adapt to the surplus of fat. This leads to triglyceride accumulation in non-adipose tissues such as the liver, muscle and pancreas, ultimately leading to malfunctioning of these organs. Adapted from (Lewis *et al.*, 2002)

As illustrated in figure 3, the belief is that in situations of continuous energy surplus, the adipose tissues cannot store the increased amount of energy as triglycerides (TG) in the fat cells. The result is that the fatty acid "spill-over" from the subcutaneous adipose depots (or perhaps also fats derived directly from the blood during the postprandial state) will eventually end up as fat in other tissues including visceral fat, liver, muscle, pancreas, heart etc. This so-called ectopic fat deposition in turn has detrimental effects on the metabolism in these tissues, and there is evidence that hepatic steatosis, intra-skeletal myocellular lipid accumulation, lipotoxicity in pancreas, deposition of fat in cardiac myocytes, and accumulation of fatty acids in the kidney are all results of this condition (Schaffer, 2003).

1.2.1 Lipodystrophy and its role in MS, T2D and CVD

The insight into the possible causal relationship between upper-body obesity and the development of insulin resistance, hyperglycaemia, dyslipidaemia, and hepatic steatosis gained support from the characterization of lipodystrophy syndromes. Lipodystrophies are clinically heterogeneous aquired or inherited disorders characterized by the selective loss of adipose tissue. Although patients suffering from these syndromes are often of normal body weight, they may suffer from a variety of different metabolic disorders, eventually leading to clinical disease. The clinical phenotype is often a selective lack of subcutaneous fat from face, arms and legs, whereas the intra-abdominal fat is maintained to some extent. The associated complications often include accumulation of triglycerides in the lever and skeletal muscle and lowered plasma adiponectin levels, all contributing to insulin resistance and increased risk of T2D and CVD (Garg, 2004). Patients suffering from lipodystrophy may actually have substantially increased metabolic rate (energy expenditure pr. day), and although they have a relatively normal BMI and are insulin sensitive at a younger age, they may eventually become insulin resistant in later life, despite still being of normal body weight (Johansen *et al.*, 1995).

Hence, this pathophysiological picture is overall in agreement with the development of lipotoxicity and the MS as illustrated in figure 3. Inherited lipodystrophies are caused by genetic defects in a number of different genes; in many cases genes involved in fat cell differentiation, e.g. *PPARγ* (Hegele, 2005). However, lipodystrophies can also be acquired: Treatment of Human Immunodeficiency Virus (HIV) using antiretroviral therapy is an example of a clinical disorder associated with the development of lipodystrophy (~40% of patients), making HIV-infected patients the most prevalent patient group with lipodystrophy (Garg, 2004). These patients have disturbed postprandial free fatty acid (FFA) metabolism, most likely due to inadequate incorporation of FFA into TG in subcutaneous adipose tissue (van Wijk *et al.*, 2005).

As obesity increases the risk of disease, it could be hypothesized that partial ablation of fat (e.g. liposuction) could improve or eliminate several of the risk factors associated with obesity. Klein et al. evaluated the effects of large-volume tumescent liposuction in obese subjects with or without T2D. The fat removed was primarily superficial and deep subcutaneous abdominal fat. Although the subjects experienced a loss of approx. 10 kg of subcutaneous abdominal body fat (keeping the amount of visceral fat unchanged) and a significant decrease in waist circumference, they had a tendency for a lower circulating adiponectin level. Also, they did not show any improvement in the insulin sensitivity of liver or skeletal muscle as assessed by the euglycemic-hyperinsulinaemic clamp technique (Klein *et al.*, 2004a). However, others have found improvements in insulin sensitivity after selective removal of less than 1 kg of visceral fat in human patients in connection with bariatric surgery procedures (Arner, 2004).

Supportive evidence has also been obtained from the use of transgenic animal models:
Reitman and colleagues reviewed the metabolic complications in mice with different degrees of fat ablation, and found that the degree of fat loss correlated with the frequency with which the mice were affected by mortality before weaning. Also the degree of fatty liver, level of hypertriglyceridaemia, and age of onset of hyperglycaemia were associated with the degree of fat-depletion (Reitman *et al.*, 1999).

1.2.2 Metabolic characteristics of different fat depots – influence on hepatic and whole-body insulin sensitivity

It is a long-standing hypothesis that hepatic insulin resistance is mediated – at least in part – by an increased flux of FFA from visceral fat to the liver by the portal circulation (Bjorntorp, 1991), and several confirmatory studies now exist. In anaesthetised dogs, the mean effective dose of insulin for suppression of lipolysis was almost fourfold higher in visceral adipose tissue than for whole-body suppression of lipolysis, suggesting that only at extremely high insulin concentrations is lipolysis suppressed in visceral fat (Mittelman *et al.*, 2002). Hence, an increased free fatty acid flux to the liver may account for hepatic insulin resistance often found in the obese state. Likewise, in moderately obese rats, surgical removal of visceral fat significantly improved hepatic insulin sensitivity compared to rats having subcutaneous fat removed, and improved insulin sensitivity was observed despite the (whole-body) plasma FFA level was unchanged (Barzilai *et al.*, 1999;Gabriely *et al.*, 2002).

In humans, the systemic levels of fasting serum TG and FFA have in some studies been identified as risk factors for the development of T2D, but other studies suggest that the abundance of visceral fat versus the same amount of subcutaneous fat does not necessarily translate into higher levels of FFA (Couillard *et al.*, 1998). However, the postprandial levels of TG and FFA are much higher in men with higher levels of visceral fat (Couillard *et al.*, 1998), or in women with a android fat distribution versus a gynoid distribution after a high fat load (Mekki *et al.*, 1999). Like in dogs, visceral obesity in humans is also associated with a decrease in the anti-lipolytic effect of insulin, as assessed via FFA turnover (Albu *et al.*, 1999). With the use of very sophisticated isotope dilution/hepatic vein catherization techniques, a recent study also concluded that the contribution of visceral adipose tissue lipolysis to the total delivery of FFA to the liver increases with increasing levels of visceral fat in humans (Nielsen *et al.*, 2004).

In humans, further support of a causal relationship between FFA and T2D comes from evidence that the drug acipimox both affects FFA and insulin sensitivity. In obese T2D and non-diabetic patients this long-acting anti-lipolytic agent lowered fasting levels of plasma FFA and plasma insulin and increased insulin-stimulated glucose uptake. It also improved the outcome of oral glucose tolerance tests (Santomauro *et al.*, 1999;Bajaj *et al.*, 2004). In T2D patients, treatment with acipimox for 12 weeks did not decrease the fasting serum levels of FFA, but decreased serum cholesterol and TG levels and improved glycaemic regulation as assessed by fructosamine and HbA1c measures (Davoren *et al.*, 1998). The long term efficacy of this drug on glucose regulation is disappointing however (Saloranta *et al.*, 1993).

1.2.3 Intrahepatic fat

Intrahepatic fat accumulation may also be involved in the development of MS, T2D, and CVD, and several clinical and experimental studies have suggested that non-alcoholic fatty liver diseases (NAFLD), in its whole spectrum ranging from pure fatty liver to non-alcoholic steatohepatitis (NASH) and cryptogenic cirrhosis, may simply be the hepatic manifestation of the metabolic syndrome (Marchesini *et al.*, 2003;Marchesini *et al.*, 2005). The causal relationship between obesity, whole-body insulin resistance and hepatic fat accumulation has however presumably been very difficult to establish, because prospective studies of hepatic fat accumulation involving even small numbers of human subjects are very difficult to carry out.
Studies in animal models do support that intrahepatic TG accumulation is indeed involved as a causal factor in the development of MS (Qi *et al.*, 2005), Also, a recent study in glucose tolerant patients compared subjects with or without hepatic steatosis assessed via ultrasound scanning of the liver. As expected, based on the inclusion criteria (only glucose tolerant patients were allowed), there was no significant differences in either circulating glucose or insulin levels two hours after glucose loading, but the homeostasis model (HOMA) insulin resistance index was significantly higher in patients with signs of steatosis and this was also independent of BMI (Venturi *et al.*, 2004).

1.2.4 Intramuscular fat

The role of intra-myocellular lipid (IMCL) accumulation for the development of MS is still not clarified. Although associations between IMCL content and insulin resistance have been reported (Pan *et al.*, 1997), others have found higher levels of IMCL in physically trained persons compared with sedentary individuals (Goodpaster *et al.*, 2001), thus questioning the pathogenic role of IMCL. It is therefore likely that increased IMCL content does not represent the direct cause of the development of insulin resistance *per se*, but is rather a marker of the presence of other lipid intermediates linked directly to defects in insulin signalling (Perseghin, 2005).

1.2.5 Animal models of the "unhealthy lean phenotype" and "the healthy obese phenotype"

One would assume that lean individuals are in general metabolically more healthy and thus without the diseases associated with obesity, such as T2D and MS. Although this may be the case in some animal models, there are also several studies of mice, which via transgenic modelling are turned lean, but still have a metabolically unhealthy phenotype. Hence, a lean phenotype is not always synonymous with a healthy phenotype, and the models described below support the hypothesis that fatty acid spill-over from fat depots, whether it occurs in lean or obese models, leads to lipotoxic effects on non-fat tissues.

The high mobility group protein HMGI-C is localized in the cell nucleus, where it functions as a transcription factor predominantly expressed during embryogenesis. *Hmgic* knockout mice gain substantially less weight without any apparent differences in food intake, compared to wild type mice. The knock-out (KO) mice have decreased fat cell proliferation and hence ~87% less fat (Anand and Chada, 2000). Unfortunately, the authors of the paper did not report any clinically relevant measures regarding nutrient metabolism, such as insulin sensitivity and hepatic lipid content. Thus, as indicated by Danforth in the associated commentary to that study: "Although manipulation with the expression of this gene, whether by genetic or medical methods may seem as a reasonable strategy, such changes in fat cell differentiation and loss of body fat may not necessarily turn into beneficial changes in whole body energy metabolism" (Danforth E Jr, 2000).
The Preadipocyte factor-1 protein (Pref-1*)* is a transmembrane protein highly expressed in preadipocytes**.** Transgenic mice with selective overexpression of Pref-1 in adipose tissue did not exhibit changes in food intake compared to wild type mice. They had however, a substantial loss of adipose tissue, and the mice exhibited hypertriglyceridaemia, impaired glucose tolerance, and decreased insulin sensitivity, thus demonstrating that inhibition of adipogenesis by pref-1 (presumably by inducing an "arrest" in adipocyte maturation) *in vivo* resulted in an impairment of adipocyte function and lead to the development of metabolic abnormalities (Lee *et al.*, 2003).
The perilipin protein is associated to the surface of lipid droplets, where it protects the droplet from being broken down by hormone-sensitive lipase (HSL). The *peripilin-/-* mouse has a constitutively activated lipolysis (via uncoupling of the HSL regulation) and increased energy expenditure. This mouse, although being very lean despite increased food intake, eventually develops insulin resistance and impaired glucose tolerance (Martinez-Botas *et al.*, 2000;Saha *et al.*, 2004).
Lipoprotein lipase is positioned at the luminal surface of the vascular endothelium where its function is to hydrolyze triglycerides from circulating chylomicrons and very low density lipoproteins (VLDL). Transgenic non-obese mice with selective overexpression of lipoprotein lipase in liver and muscle, developed insulin resistance in liver and muscle, likely due to accumulation of intracellular fatty acid-derived metabolites in the insulin resistant tissues (Kim *et al.*, 2001). These studies of lean, but metabolically adversely affected mice models underscore the importance of abnormal lipid deposition in the development of insulin resistance.

On the contrary, one would assume that animal models with an obese phenotype would have increased risk of metabolic abnormalities. However, there are models that are actually obese, but have a risk profile that is equal, or even better than the one for wild type animals.
The adipocyte lipid-binding protein (AFABP) is localized in the cytoplasma where it binds both long chain fatty acids and retinoic acid thus controlling lipid transport in adipocytes. The *AFABP-/-* mouse

exhibits reduced fatty acid efflux from adipose tissue, possibly via downregulation of the activity of hormone sensitive lipase. Despite increased adiposity, the KO mouse has lower fasting blood sugar levels and increased insulin sensitivity and improved glucose tolerance compared to wild type animals (Uysal *et al.*, 2000;Baar *et al.*, 2005).
Lipin-1 (Fatty liver dystrophy protein), encoded by the *Lipin1* gene is a nuclear protein, known to be involved in adipocyte differentiation. Specific overexpression of lipin-1 in adipose tissue increased the amount of body fat tissue, but the insulin sensitivity was improved compared to wild type animals. This was likely induced by an increased triglyceride synthesis and storage in adipose tissue, preventing fatty acid overflow and ectopic fat storage in muscle, liver or pancreas. Conversely, specific overexpression of lipin-1 in skeletal muscle induced insulin resistance (Phan K and reue k, 2005).
The Diacylglycerol O-acyltransferase 1 protein (DGAT) encoded by the *Dgat1* gene is an integral membrane bound protein residing within the endoplasmic reticulum. The protein catalyzes the terminal and only committed step in TG synthesis by using diacylglycerol and fatty acyl CoA as substrates. Transgenic mice overexpressing *DGAT1* were more obese (had ~20% more body weight) but had normal glucose and insulin tolerance tests, and normal triglyceride deposition in skeletal muscle and liver (Chen *et al.*, 2002).
The phosphoenolpyruvate carboxykinase protein (PEPCK) encoded by the *Pepck* gene is a rate-limiting gluconeogenic enzyme. Transgenic mice with selective overexpression of PEPCK in adipose tissue developed obesity, but did not develop insulin resistance (Franckhauser *et al.*, 2002).
The adiponectin protein is encoded by the *Acrp30* gene, and it is synthesized exclusively by adipocytes and known to be secreted into plasma. Mice with transgenically induced higher levels of adiponectin had increased fat mass but had also improved lipid clearance and improved suppression of insulin-mediated endogenous glucose production (Combs *et al.*, 2004).

Although the ectopic fat hypothesis may seem relatively straightforward, there are reports of transgenic animals with - what seems to be – a selective decrease in white adipose tissue via manipulation of either insulin receptor in fat tissue (Bluher *et al.*, 2002), heterozygotic Crebbp mice (a nuclear enzyme involved in histone acetylation during transcriptional activation) (Yamauchi *et al.*, 2002) and increased expression of the transcription factor FOXC2 expression (Cederberg *et al.*, 2001), and which also have improved insulin sensitivity. However, these studies lack a thorough description of food intake, so the (if any?) effects on the central appetite regulation cannot be ruled out.

Overall, the above discussed animal models support the accumulating evidence that peripheral subcutaneous fat functions as fat depots, effectively absorbing fat from the blood and maintaining this fat in depots. Also, without these fat depots, the clearance of fats and nutrients from the blood is attenuated, potentially increasing the load of these nutrients toward other tissues such as muscle, liver and pancreas, eventually leading to malfunction of these tissues.

1.2.6 Definition of a healthy lean phenotype with optimal metabolic regulation for the interpretation of future risk of MS, T2D and CVD.

The human body is genetically able to adequately regulate blood pressure, body temperature, salt balance etc. in order to maintain its homeostasis. Hence, the body is usually also perfectly adapted to regulate the level of glucose, lipids and amino acids in the blood in order to supply the tissues and organs with the nutrients necessary for the provision of energy and for cell maintenance or cell growth. Under normal circumstances the body adjusts this regulation under a wide variety of conditions such as during sleep, starvation and high intensity exercise. Also overfeeding is regulated in the sense that excess energy is efficiently stored as triglycerides in fat depots, where the lower-body, subcutaneous fat depots seem metabolically best adapted for this purpose. Chronic perturbations of energy balance are less likely to be optimally regulated however, as these fat depots cannot expand infinitely. Thus, chronic oversupply of energy may lead to a state where the nutrients

are not properly taken up by the tissues, and there is risk of so-called “leak” or “spill-over” of energy from these tissues. In such situations, the level of nutrients in the blood is not adequately regulated. To avoid such situations, the body engages counter-regulatory mechanisms. In humans, there are suggestions that hormones such as leptin and/or possibly adiponectin may be counter-regulatory hormones, leptin acting as both a satiety signal in the brain, and as a fat-burning signalling directed to peripheral tissues such as liver and muscle tissues (Unger, 2005). However, as is unfortunately often seen, innate counter-regulatory signalling may not be sufficiently powerful to regulate such chronically imbalanced states.

Many such imbalances are reversible by behavioural means, e.g. lowering of energy intake and concomitant weight loss may help the body to better regulate the metabolism of nutrients.

Also, at least theoretically, drug-induced changes in nutrient flux – whether regulated on the transcriptional or on the protein level – may be used as a mean for re-establishing nutrient regulation. As previously illustrated and as it will be discussed in the case of CLA supplementation, the method by which such energy imbalance is regulated may not be equally effective in terms of overall health effects. Thus, although one can make suggestions of plausible useful treatment based on biological grounds, in any case, real experiments need to be performed, and it seems highly important to evaluate if metabolic changes are beneficial not only in terms of the study efficacy endpoint (e.g. fat loss), but also in terms of other risk factors related to the medical condition (e.g. hepatic steatosis or insulin sensitivity), and preferably also to other conditions.

1.3 Molecular genetic aspects in the pathogenesis of obesity

Traits like eye or hair colours are substantially influenced by genetic inheritance. Likewise, almost every human trait is to some extent genetically determined, but where some traits may be determined by a single causative gene (monogenic), others may be under the influence of a variety of different and interacting susceptibility genes (polygenic) as well as epigenetic and environmental factors.
The basic premise of the studies of obesity genetics is the assumption that the inherited component of obesity is real and that is has a relatively large impact on the development of obesity, also compared to non-genetic factors such as environmental factors (physical activity, socioeconomic status, diet etc.) and epigenetic factors (non-mendelian, paternally or maternally inherited traits, that is not ascribed to changes in the nucleotide sequence, but to subtle modifications of the genes, which in turn may affect their expression). Thus a low genetic heritability will decrease the power of the study, and the likeliness of finding a genetic influence will diminish. Heritability can be estimated using either twin studies (looking for greater concordance between monozygotic twin pairs than between dizygotic twin pairs), by studying families (examining the degree of similarity within families versus between families) or by adoption studies (looking for greater concordance between biological relatives than for the adoption parents-adoptive child relationship). A complementary statistic is λ_s, which is the degree of elevated risk of disease for a sibling of an affected individual compared with a member of the general population (see box below).

$$\lambda_s = \frac{\text{Risk of disease for sibling}}{\text{Risk of disease for background population}}$$

λ_s does not exclusively reflect pure genetic influence, but it also reflects the impact of familial environmental factors. However, before embarking on a costly study, one should have prior evidence that genetic variation plays some role in determining the phenotype, and it is also relevant to have an estimate of heritability or λ_s.
For T2D, for which genetic influence have been quite intensively studied, several estimates of heritability have been suggested. Florez et al. describe, that in T2D, the absolute risk to siblings is 30-40% as compared to a population prevalence of 7%, providing a releative risk to siblings of four to sixfold (Florez *et al.*, 2003). Other investigators argue that the relative risk (λ_s) is about 3 (Parikh and Groop, 2004), while yet others suggest that heritability estimates of T2D are unrealistic to derive, as many genetic and environmental effects are intimately related and interact throughout life (McCarthy, 2004).
For obesity, different measures may be defined e.g. BMI, body fat percentage, waist circumference, visceral fat etc. The correlation between each of these measures is in general very variable. Hence, strictly speaking, one should actually talk about several obesity phenotypes. Therefore, it can also be argued that any individual can be susceptible to develop a higher BMI, but not necessarily a higher visceral fat content. Most genetic studies have looked at BMI as the "obese phenotype".
The results from the studies of the heritability of common (common = polygenic) obesity (e.g. BMI) have generally been quite heterogeneous, the heritability level being the highest with twin studies and the lowest in adoption studies. When several types of relatives are used jointly in the same design, the heritability estimates typically cluster around 25-40% of the age- and gender-adjusted phenotypic variance (Claude Bouchard *et al.*, 2004).
If considering which obesity phenotype would be most important for metabolic complications, one would most likely study the amount of visceral fat content. But measurement of visceral fat content is limited by a very laborious and costly procedure, and therefore only very few studies have looked at genetic susceptibility for visceral fat accumulation. Reliable estimates of the genetic contribution of visceral fat content can consequently not yet be made.

The study of the genetics of human obesity has focused on both monogenic (including syndromic) and polygenic obesity, and a variety of different study designs, including Quantitative Trait Loci

(QTL), linkage studies and association studies as well as the use of a lot of different transgenic and knockout animal models have been used, recently reviewed by Bell et al. (Bell *et al.*, 2005).

1.3.1 Monogenic and syndromic obesity

From the beginning of the 1990s, the development of genome-wide genetic maps (including the sequencing of the human genome) permitted the widespread application of genome-wide linkage analysis to disease status. When applied to complex traits, this method has proved to be largely unsuccessful, although some genes contributing to complex diseases have been identified.
On the contrary, for analysis of single-gene disorders, linkage analyses have been tremendously successful, leading to many new insights into molecular disease pathogenesis, including obesity.
In general, a distinction is made between monogenic obesity and syndromic obesity, the latter being phenotypically characterized by several distinct clinical abnormalities of which mental retardation is a common one. Within the syndromic phenotypes, the most well-known are Prader-Willi, Cohen, Alström and Bardet-Biedl syndromes. The monogenic obesity phenotypes are usually related to disturbances of the central regulation of appetite regulation (Clement, 2005). Thus, within monogenic phenotypes, the most studied variants have been identified in the genes encoding leptin, leptin receptor, pro-opiomelanocortin (POMC), proconvertase 1 and melanocortin-4 receptor (MC4R), all genes which are related to the central regulation of appetite (Clement, 2005). The importance of these genes for the development of obesity has in most cases been confirmed by knock-out or transgenic animal models. In humans these mutations are rare. However, there are suggestions that mutations in the gene encoding MC4R may be the most common form of known monogenic obesity accounting for 2-3 % of juvenile-onset obesity among Danes (Larsen *et al.*, 2005a).

1.3.2 Polygenic obesity

It is generally agreed that common obesity (both defined as a discrete and as a quantitative trait) has a complex pathogenesis, with multiple genetic and environmental factors contributing to the observed phenotype (e.g. BMI, waist circumference etc.). Because of its multi-factorial nature, each individual genetic variant generally has only a modest effect, and the interaction of genetic variants with each other or with environmental factors can potentially be quite important in determining the observed degree of obesity. Yet it remains largely unknown which genetic variants explain the inherited variation of obesity. Genetic association studies, in which the allele or genotype frequencies of markers are determined in affected individuals and compared with those of control subjects (either population- or family-based), may be an effective approach to detect the impact of common variants with modest effects (Newton-Cheh and Hirschhorn, 2005). With the recent increase in single nucleotide polymorphism (SNP) discovery and genotyping technologies, large-scale association studies have become feasible, and small-scale association studies have become plentiful. According to the yearly update on the overview of studies of obesity genetics, association studies between gene variation and obesity phenotypes now represent positive associations with more than 358 findings in 113 different genes (Perusse *et al.*, 2005). However, despite the accumulating number of "positive studies", there have been very few gene variants, where several independent studies have been supportive, thus questioning the validity of the majority of these small positive association studies, most of which are statistically underpowered.

The polygenic inheritance of obesity has been described by different hypotheses:
1) The Common Disease/Common Variant (CD/CV) hypothesis predicts that the genetic risk for common disease (or obesity) will often be due to disease-predisposing alleles with relatively high frequencies – that is, there will be one or a few predominating disease alleles at each of the major underlying disease loci (Lander, 1996;Reich and Lander, 2001).

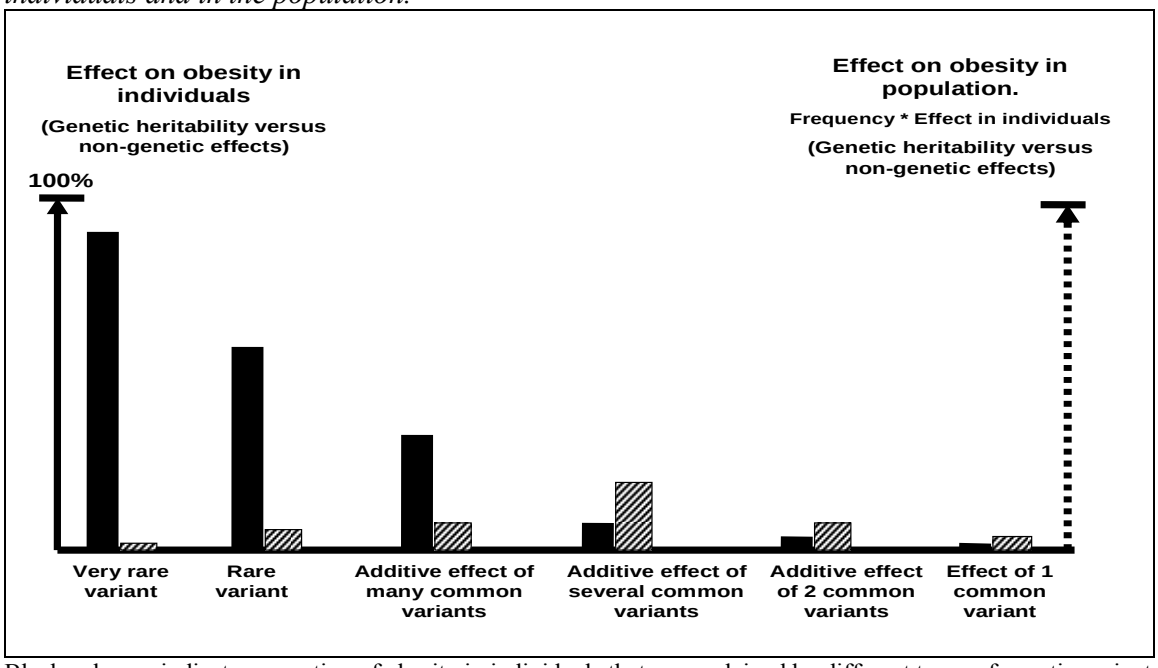
2) A hypothesis, in which a large number of loci, each with multiple disease-predisposing alleles of low frequency (high allelic heterogeneity) is involved in the increased risk of obesity (Pritchard, 2001;Pritchard and Cox, 2002).

3) The common variants/multiple disease hypothesis. The hypothesis states that the common alleles which contribute to a given disease under a certain combination of interacting genes and environmental conditions, may act in other genetic backgrounds influenced by other environmental factors, resulting in different but possibly related clinical outcomes. For example, physical inactivity is a common risk factor to both T2D and obesity. Both disorders are influenced by different diets, both may share loci determining carbohydrate and lipid metabolism, ultimately contributing to disease (Becker, 2004). Formally this hypothesis does not refute any of the other 2 hypotheses.

Also, none of the three hypotheses exclude that each of several genetic variants may only have a small or moderate effect on obesity, and that the cumulative contribution of each or all of these variants may only become important when there is an interaction with environmental factors predisposing to their phenotypic expression, such as overeating and reduction in physical activity.

Currently it is assumed that approximately 90 % of the sequence variation among human individuals is due to common variants (with allele frequency ≥ 1%), and the remaining 10% is due to a vast array of variants that are each rare in the population (HapMap Consortium, 2003). A haplotype is defined as a specific set of alleles observed on a single chromosome.
An example of a haplotype is the coexistence of the three nucleotides -1505A, -1881T and -2604C at the three positions: -1505, -1881 and -2604 in the *PPARγ2*.
The goal of the ongoing International HapMap project is to determine the common patterns of DNA sequence variation in the human genome. The project will develop a map of these patterns across the genome by determining the genotypes of one million or more sequence variants, their frequencies and the degree of association between them. The map will be based on DNA samples from populations with ancestry from Africa, Asia and Europe. One of the project aims is to allow the discovery of sequence variants that affect common disease to improve diagnostics and therapeutic intervention (HapMap Consortium, 2003). However, the success of the HapMap project to identify disease variants highly depends on the assumption that the "common variant/common disease" hypothesis is true, because many rare variants will not be detected. Unfortunately there are several arguments against this hypothesis. Generally, gene variants which only have marginal effects on the phenotype are more likely to be maintained and therefore have a higher frequency. Gene variants with moderate effects are more likely to be sorted out due to evolutionary selection pressure, and gene variants with fatal effects will in most cases not be passed on to the next generation. Therefore, the more common the variant is, the less likely it is to have any impact on common diseases. In contrast, rare variants are more likely to have large effects on disease. Rare variants will of course per definition not give rise to common disease. It can be argued that the genetic evolution in western countries is characterized by an absence of strong natural selection. Hence, there is likely to be a broad spectrum in frequency of gene variants, many of which are likely to be common in the population. Each common variant does not significantly increase the risk of being obese, and it does not have any impact on obesity in the population. However, there may be a subset of individuals who possess several variants that together increase their risk of obesity. Also, the totality of such people may affect obesity in the whole population. Conversely, the number of people with many such common variants will be small (illustrated in figure 4 below).

Figure 4: Illustration of the effects imposed by rare and by common genetic variants on obesity in individuals and in the population.



Black columns indicate proportion of obesity in individuals that are explained by different types of genetic variants.
Hatched columns indicate proportion of obesity in the population that are explained by different types of genetic variants.

The candidate gene approach used for association studies can be based on various evidence; the knowledge of the function of the gene under consideration and the anticipation that changes in the gene sequence, either changing the function or the amount of the gene product, may influence the phenotype under study. Thus, the choice can be based on the physiological role of its encoded protein product in the mechanism of obesity, the consequences of its genetic deletion (knock-out) or its overexpression (transgenesis) in the rodent and even the *in vitro* functional characteristics of mutations or variations of the DNA studied (biological candidate).
Other evidence can be the chromosomal location, i.e. if positioned in a region previously shown to be genetically linked to obesity in man or animals (positional candidate).

In common diseases (and common obesity) with a polygenic inheritance, common genetic variants (or polymorphisms defined as those for which two or more alleles each exist in 1% or more of the population at large), are individually or together *not* expected to be necessary nor sufficient to cause the disease. That is, some individuals will have the variant but not the disease, and others will have disease without harbouring a causal variant of that gene. Because of this imperfect correlation, association studies must compare frequencies of a putative causal variant in individuals with disease and in appropriate control subjects. If the genetic variant is found at a statistically significant higher frequency in affected individuals, this implies that the variant is associated to disease.
Proof of causality of a gene variant-phenotype association typically depends on several lines of evidence. First, for polygenic diseases, the putative causal genomic changes should be found *more* often in affected individuals. Second, one hopes for a "smoking gun", – i.e. that the disease-associated variants are obviously deleterious to protein function (due to truncation or deletion of a coding region or alteration of a highly conserved residue) or expression (due to changes in important regulatory regions). Success is typically declared when the following criteria are all satisfied: The putative disease gene (or genes) 1) is located in a chromosomal region that co-segregates with disease in affected families, 2) contains multiple independent variants that are perfectly (or almost) associated with disease status in the families, 3) obviously alter protein function or expression (Hirschhorn and Altshuler, 2002). Confirmation of the satisfaction of these criteria in independent studies will further strengthen the association.

Although various association studies have suggested that individual genetic variants have important effects on BMI, and although genome-wide studies have also suggested that various loci do show some associations to obesity, there are yet no convincing evidence (i.e. independent replicated studies) that any individual gene variant or any individual locus has a significant effect on common obesity (Bell *et al.*, 2005;Perusse *et al.*, 2005). Actually, the PPARγ Pro12Ala variant is very likely to be the variant most often associated to obesity, but even this association is not yet definitive as it will be discussed further in section 1.4.3.

1.4 PPARγ – is it a central player in obesity and related diseases?

The peroxisome proliferator-activated receptors (PPARs) comprise a subfamily of the nuclear hormone receptor (NHR) superfamily. The family of PPARs includes the PPARγ, PPARα and PPARβ. All PPAR family members serve as transcription factors via heterodimer binding with the retinoic acid receptors (RXRα, RXRβ og RXRγ). The PPARs are all activated by a variety of fatty acids, but the specificity for fatty acids differs between the PPARs. They also differ in their tissue expression, PPARα being primarily expressed in the liver, PPARβ being expressed in virtually all tissues and PPARγ being mostly expressed in adipose tissue.

The PPARα is known to play an important role for plasma TG regulation and the fibrate drugs, which are used in clinic care of dyslipidaemia are known to decrease TG levels via their action on PPARα in the liver. The exact role PPARβ has not yet been firmly established, but there is evidence that it is involved in cholesterol metabolism in the liver (Larsen *et al.*, 2003b).

It is well established that the PPARγ protein has a very important role in adipocyte differentiation, but it has also been reported to be expressed in a variety of different tissues. It is expressed as the two different transcripts PPARγ1 and PPARγ2, giving rise to identical proteins except that the PPARγ2 proteins carry an extra 28 amino acid long n-terminus. Maybe the existence of tissue-specific expression and different transcripts may have important consequences for its biological importance. To address the importance of variation within the *PPARγ* and the associated effects on obesity and related diseases, it is therefore important to evaluate its role in various human organs like fat tissue, liver, muscle and pancreas - all organs that have a major influence in the pathogenesis of obesity and related diseases. The following sections will address the biological function of PPARγ and of each of the transcripts PPARγ1 and PPARγ2 in relation to cellular functions, and it will present evidence from animal and human studies, including the possible role of gene variants in the promoter region as well as the coding on human obesity, carbohydrate and lipid metabolism, and the development T2D and CVD. It will be discussed whether there is consistency between the current biological knowledge and the findings from genetic studies.

1.4.1 The importance of the PPARγ receptor for fat cell differentiation and metabolic regulation.

As described in the review, paper 3 (Larsen *et al.*, 2003b), our understanding of the PPARγ and its involvement in adipocyte development has increased substantially over the recent years. The interest is – at least in part – explained by the accumulating evidence that PPARγ agonistic activation using the Thiazolidinedione drug class or similar drugs, can be used as a treatment modality to attenuate the insulin resistance which seems to underlie the development of T2D in the majority of patients. It is generally agreed that prevention of insulin resistance would be a better solution to tackle the disease, rather than treating the disease with other means when the disease is more manifest and often followed by complications. Hence, as the insulin resistance is thought to underlie the development of T2D, the underlying mechanisms behind these insulin-sensitizing effects of PPARγ activation is of major interest for the medical industry as well as for the scientific community as it may give further insight into the pathophysiological mechanisms behind the development of the disease. Also, this insight can be useful for the development of other treatment options, including medical drugs but potentially also novel herbal compounds and dietary supplements or maybe a better optimization of the diet.

The importance of PPARγ has been investigated by using a variety of approaches, including transgenic mice. As discussed in the review paper, several independent research groups have generated and investigated mice heterozygous for *Pparg* (Larsen *et al.*, 2003b). However, possibly due to different genetic background of the mice strains used, and due to different study designs, such as length of study (i.e. also the age of mice), the results of the characterization of these mice are not always congruent.

Initial studies suggested that these mice exhibited greater insulin sensitivity and were protected from the development of insulin resistance mediated by a high fat diet (Kubota *et al.*, 1999;Miles *et al.*, 2000). A subsequent study confirmed that when fed a high fat diet, these mice are less obese, less

insulin resistant, but that they have only a normal glucose tolerance, probably mediated by a less than expected insulin release because of an increased TG content in the beta cells (Matsui *et al.*, 2004). Of further contrast, another group found that *Pparγ* +/- KO mice also develop insulin resistance when fed a high fat diet (Miles *et al.*, 2003).

Transgenic mice with a dominant negative *pparγ* hL466A mutation leads to impaired adipocyte differentiation and lipodystrophy. They do not develop severe insulin resistance when fed normal diet, but overt insulin resistance is induced by long term high fat feeding (Freedman *et al.*, 2005). As is often the case, the identification of these phenotypes were a result of genotype-environment interactions. Hence, genetic manipulations or natural genetic variations may in many cases only result in a different phenotype, when studied under certain conditions, such as high fat feeding.
The understanding of the biological function of PPARγ has also been quite intensively studied using transgenic mice with selective tissue *Pparg* inactivation. Hence there is now quite a few different *Pparg* transgenic mice, i.e. fat tissue specific deletion of *Pparg* (both g1 & g2) (He *et al.*, 2003;Imai *et al.*, 2004;Jones *et al.*, 2005), specific deletion of *Pparg2* (Koutnikova *et al.*, 2003;Zhang *et al.*, 2004;Medina-Gomez *et al.*, 2005), liver specific deletion of *Pparg* (both g1 & g2) (Gavrilova *et al.*, 2003;Matsusue *et al.*, 2003) and muscle specific deletion of *Pparg* (both g1 & g2) (Hevener *et al.*, 2003;Norris *et al.*, 2003).
A recent comprehensive review (Argmann *et al.*, 2005) (and to which I agree!) of the main *Pparg* tissue specific KO mice models that have been described in the recent years, suggested that in the models with selective absence of WAT, the liver PPARγ participates in both fat regulation and glucose homeostasis, but in the models with presence of WAT, the impact of PPARγ in the liver on glucose homeostasis is rather minimal. The role of PPARγ in the muscle is however, not yet obvious, as two independent reports of muscle-specific *Pparg* KO mouse models were essentially opposite, one study suggesting that mice lacking muscle PPARγ develop severe muscle insulin-stimulated insulin resistance and as result are hyperinsulanemic, glucose intolerant and hypertriglyceridaemic (Hevener *et al.*, 2003), whereas the other study suggested that muscle-specific PPARγ KO mice have normal glucose homeostasis and insulin levels, but have reduced hepatic insulin sensitivity, suggested to be a consequence of the increased WAT mass (Norris *et al.*, 2003). Further, although some studies suggest that muscle PPARγ is a direct target of TZDs, most studies suggest that PPARγ in muscle is more responsible for coordinating the use of energy rather than directly controlling glucose homeostasis or responses to insulin, validating the concept that WAT is predominantly responsible for the insulin-sensitizing effect of PPARγ (Argmann *et al.*, 2005).

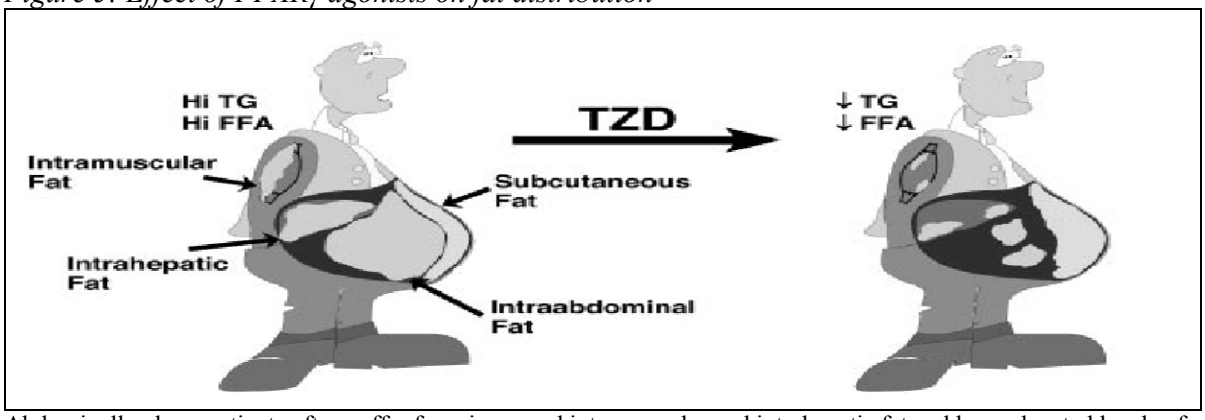
As the amount and activity of visceral fat seems to be one of the key factors in the regulation of hepatic, and probably whole-body glucose regulation, one of the key questions is therefore the actual role of PPARγ in the differentiation of (lower-body) subcutaneous fat versus (upper body) visceral fat. Our current understanding of PPARγ´s interaction in fat cell differentiation is, however, still quite complex. Studies in mice give evidence that PPARγ activation of the lipogenic programme does not depend on the presence of glucocorticoids, and inasmuch as rodent inguinal and retroperitoneal fat can be taken as representatives of "human" subcutaneous and visceral fat, PPARγ activation seems to promote differentiation of subcutaneous fat to a higher degree than of visceral fat (Berthiaume *et al.*, 2004). Others have concluded that the adipose-depot specific effects of PPARγ agonist cannot be explained by differences in the mRNA expression of total PPARγ between adipose tissue from human subcutaneous and omental adipose depots as the transcription levels were equal (Montague, 2002). In obese women, in both subcutaneous and visceral fat, the expression of *PPARγ2* was substantially more expressed (>20 fold) than that of *PPARγ1*. However, only *PPARγ1* was differently expressed, its levels in subcutaneous adipose tissue being approximately 2 fold those in visceral adipose tissue (Giusti *et al.*, 2003). In obese rats, treatment with a PPARγ agonist for 3 weeks significantly increased subcutaneous fat, but decreased visceral fat (Laplante *et al.*, 2003), and similar findings have been reported in human studies (Larsen *et al.*, 2003b). On the other hand, excessive – and thus toxicological - doses of PPARγ agonists in rats and monkeys induce massive lipid accumulation, oedema and anaemia (Aleo *et al.*, 2003)

In mice, selective disruption of *Pparg2* is not lethal (whereas combined *Pparg1* and *Pparg 2* KO is). *Pparg2* KO mice are of normal body weight, but have reduced WAT, less lipid accumulation and decreased adipogenesis in adipose tissue, though with no severe metabolic perturbations on 12-15 weeks on a regular chow diet. Yet, the increase in body fat is more severely compromised when put on a high fat diet (Zhang *et al.*, 2004). These findings were corroborated by another research group, although the amount of WAT was less affected by *Pparg2* disruption (Medina-Gomez *et al.*, 2005). Transfection of *Pparg* -/- mice embryonic fibroblasts shows that PPARg2 is a more powerful inducer of adipogenesis of WAT than PPARg1 (Zhang *et al.*, 2004).

Although some studies have suggested that PPARγ antagonists could be used in the treatment of obesity and MS and selective PPARγ antagonists have now been available for more than 5 years, only one *in vivo* study in mice has been carried out (and reported) using these drugs. Treatment with the PPARγ antagonist SR-202 reduced body mass, fat mass and seemed to improve insulin sensitivity in growing diabetic ob/ob mice (Rieusset *et al.*, 2002). In this study, however, there was no data on concurrent changes in energy expenditure, energy intake and levels of triglycerides in plasma and ectopic fat deposition in the liver, findings which may have occurred. Also, no similar studies are available in adult and weight stable mice, nor are there any studies in larger models such as pigs, dogs or monkeys, thus questioning whether these findings are reproducible.

Activation of PPARγ with drugs are used clinically to treat type-2 diabetes, and post-approval studies have added further evidence that the drugs currently available, pioglitazone and rosiglitazone really are (equally) effective at blood sugar regulation and insulin sensitization, although they may differ somewhat in their effects on circulating triglycerides, HDL- and LDL-cholesterol, all effects being in favour of pioglitazone (Goldberg *et al.*, 2005).
Our understanding of the mechanism of action has also improved substantially. In T2D patients, 12 weeks of treatment with the PPARγ agonist pioglitazone increased the expression of several, though not all, lipogenic enzymes in abdominal subcutaneous fat (Bogacka *et al.*, 2004). The same study found that a 24 week treatment period induced a weight gain of ~3.5 kg, of which fat tissue accounted for almost all of the weight gain. The amount of visceral fat tissue did however not change. A prior study with 24 weeks treatment of 400 mg/day of troglitazone showed decreased ratio of visceral fat/subcutaneous fat, together with preserved fasting insulin secretion and decrease of liver fat (Katoh *et al.*, 2001). Overall, these effects on body fat redistribution by activation of PPARγ activation is illustrated in figure 5.

Figure 5: Effect of PPARγ agonists on fat distribution



Abdominally obese patients often suffer from increased intramuscular and intrahepatic fat and have elevated levels of circulating triglycerides and free fatty acids. Treatment with PPARgamma agonists thiazolidinediones (TZD) decreases triglycerides and free fatty acids and induces a relative redistribution of fat – i.e. the amount of subcutaneous fat is increased, whereas the amount of visceral fat is decreased. Adapted from Bays H et al.(Bays *et al.*, 2004)

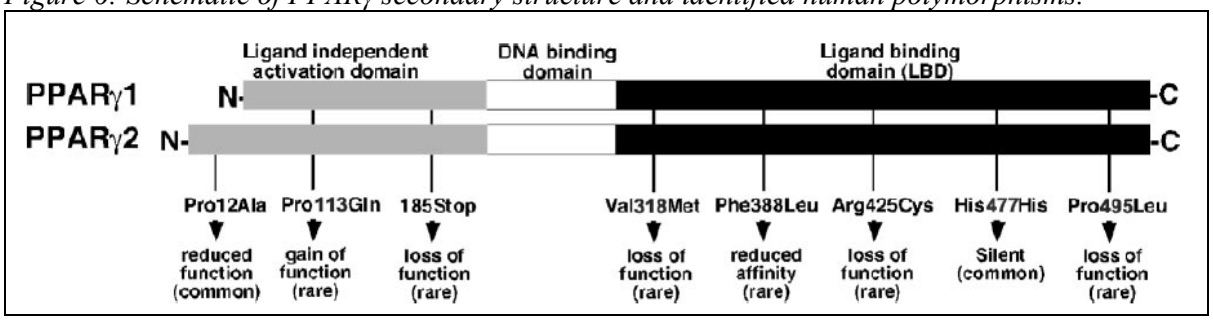
A study in T2D patients, with 16 weeks of 8 mg/day of rosiglitazone resulted in decreased liver fat content, lowering of hepatic insulin resistance, and increased expression of lipoprotein lipase (LPL) and adiponectin in subcutaneous abdominal adipose (Tiikkainen *et al.*, 2004). In addition to decreased liver fat content, a lowering of triglycerides in skeletal muscle has been reported after 12 week treatment (Mayerson *et al.*, 2002).
Also, long-term treatment (2 year) with pioglitazone compared with the insulin-secretagogue gliclazide, showed that pioglitazone was significantly more effective in long-term blood sugar regulation and on indices of insulin resistance and beta-cell function (Tan *et al.*, 2005).
Whether treatment using PPARγ agonists also can lead to improvement of CVD risk profile – in addition to the effects on insulin sensitization and glucose regulation – is now one of the major questions that are being clinically evaluated in ongoing studies, though preliminary evidence is available; in a 24 week study with non-diabetic men with coronary heart disease, treatment with 8 mg/day rosiglitazone reduced insulin resistance, and also the levels of C-Reactive Protein (CRP) and von Willebrand factor (markers of inflammation and endothelial activation): However, the treatment actually increased circulating LDL and triglyceride levels, and did not seem to translate into positive improvements in endothelial function (Sidhu *et al.*, 2004). Another study found that pioglitazone, when given as 45 mg/day to diabetic patients, improved plasma glucose and insulin levels, and in addition improved some (serum concentrations of LDL, hsCRP, MMP-9, MCP-1, HDL, adiponectin), but not all markers of CVD risk (no effects on e.g. serum concentrations of ICAM, VCAM, fibrinogen, von Willebrand, PAI-1). Interestingly though, there was an improvement in carotid intima media thickness (IMT) (Pfutzner *et al.*, 2005). Thus, as preliminary evidence on the long-term effects of PPARγ activation for the treatment of CVD is contradictory, we await further clarification from ongoing studies.

1.4.2 Genetic variation in the *PPARγ* locus

As discussed in the previous section, there is accumulating evidence that PPARγ is strongly involved in fat cell differentiation. Also animal studies and human clinical studies suggest that PPARγ activity regulates whole-body fat content, fat distribution, glucose and lipid metabolism and affects the risk of metabolic diseases. Hence, there is biologically plausible evidence that also variation in the gene encoding the PPARγ transcription factor may have important implications for obesity and related diseases. As illustrated in figures 6 and 7, quite a few different variants have been identified in *PPARγ*. But most previous studies have focused on variation within the coding region of the gene. Several of the variants identified in the coding region are missense mutations with effects on *in vitro* transcriptional activity. Some of them have been suggested to underlie clinical cases of lipodystrophy syndromes, suggesting a severe attenuation of PPARγ activity and of development of fat tissue. However, the genetic variants underlying the development of lipodystrophy are very rare (Hegele, 2005).

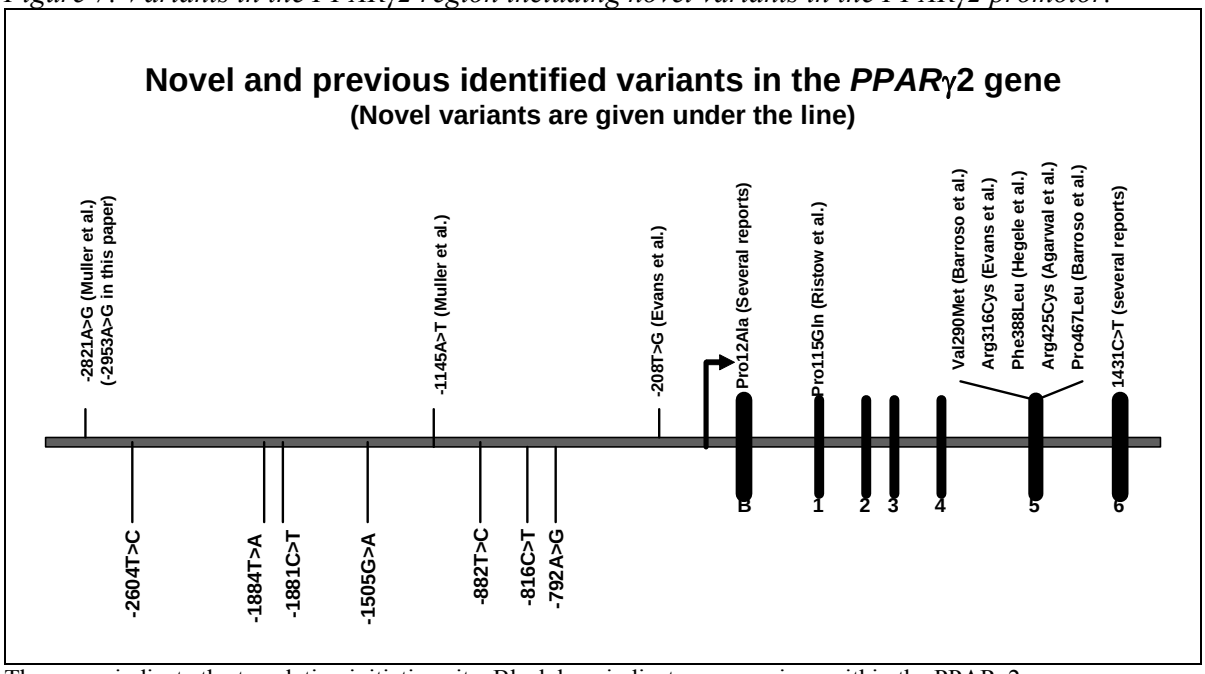
The 2 common variants; the silent 1431C>T (His477His) variant positioned within the ligand binding domain, and the Pro12Ala variant positioned in the proximal part of the ligand independent activation domain of the PPARγ2 protein are (i.e. this polymorphism does not exist in the *PPARγ1* transcript), possibly due to their high allele frequency, the most intensively studied variants in *PPARγ*. Thus the effects of these variants have been the focus in many association studies in a wide variety of human populations.

Figure 6: Schematic of PPARγ secondary structure and identified human polymorphisms.



Schematic of PPARγ secondary structure and human polymorphisms. The three principal domains of PPARγ, the N-terminal domain (*gray*), DNA-binding domain (*white*), and C-terminal ligand-binding domain (*black*), are highlighted. PPARγ1 is widely expressed, whereas PPARγ2 is restricted to adipocytes. PPARγ2 contains an additional 30 residues at its N terminus in humans (28 in mice). The common Pro12Ala polymorphism is within these extra residues of PPARγ2. All other mutations affect both isoforms. The constitutively active Pro113Gln mutation is located near serine 112 and interferes with phosphorylation. All the dominant-negative mutations are found in the ligand-binding domain and may interfere with ligand binding and/or heterodimerization with RXRα. No mutations have yet been found in the DNA-binding domain. Adapted from (Knouff and Auwerx, 2004)

Figure 7: Variants in the PPARγ2 region including novel variants in the PPARγ2 promotor.



The arrow indicate the translation initiation site. Black bars indicate exon regions within the PPARγ2 gene. Adapted from paper 1 (Larsen *et al.*, 2005b)

1.4.3 Heterogeneous findings in studies of *PPARγ* polymorphisms.

As with genetic studies of other common diseases with a complex inheritance where results are often inconsistent and therefore the subject for much dispute (Editor, 1999;Ioannidis *et al.*, 2001;Dahlman *et al.*, 2002;Terwilliger *et al.*, 2002), the available studies on the genetic influence on common obesity are often inconsistent or even contradictory (Hirschhorn *et al.*, 2002;Lohmueller *et al.*, 2003;Perusse *et al.*, 2005). For a given polymorphism the effects reported are sometimes uncertain, even conflicting, and illustrate the complexity of obesity as well as the statistical difficulties often associated with the lack of power of the analyses (Clement, 2005).

This is also the case regarding variants in *PPARγ* and its effects on T2D and obesity (Meirhaeghe and Amouyel, 2004). Still, some argue that the overall evidence actually suggests that the *PPARγ* Pro12Ala variant actually causes a modest but real effect on the risk of T2D, the Ala allele being protective compared to the Pro variant (Lohmueller *et al.*, 2003).

Recently Meirhaeghe and Amouyel reviewed the literature on common *PPARγ* variants (Pro12Ala & C1431T variants) and their association to different phenotypes, including T2D and obesity. Overall,

the data for the effect of the Pro12Ala variant remains controversial (Meirhaeghe and Amouyel, 2004) (Table 1). The review considered only studies of BMI, and to my knowledge, there are no studies available on the association between the Pro12Ala variant and visceral fat content. The *in vitro* studies of the Pro12Ala variant were quite consistent, all 3 available studies suggesting that the Ala variant had a reduced *PPARγ* transcriptional activity. But when studied *in vivo*, three independent research groups found that the insulin sensitivity was higher in Ala carriers (Meirhaeghe and Amouyel, 2004). One study evaluated the expression of PPARγ target genes in various fat depots of massively obese humans being either Pro12 or Pro12Ala carriers, and concluded that the polymorphism had only minor influence on the expression of these genes (*PPARγ2, PPARγ1, LPL* and others) (Kolehmainen *et al.*, 2003).

Table 1: Influence of the Ala12 allele on type-2 diabetes risk, insulin sensitivity and BMI

Phenotype	Number of studies with protective effect	Number of studies with deleterious effect	Number of studies with no effect
Type 2 diabetes	7	3	8
Insulin sensitivity	15	0	8
BMI	6	10	25

Modified from (Meirhaeghe and Amouyel, 2004)

The inconsistencies in the association studies can be ascribed to several factors, all essentially ascribed to three causes:
1) A false-positive association is correctly not replicated; 2) A correct report of a true association fails to be replicated in an underpowered study (false negative); 3) A true association in one population is not true in a second population because of heterogeneity in genetic or environmental background.
In brief, **false positives** can arise due to inappropriate use of the p-value of <0.05 as a criterion for declaring success, particularly if not taking into account the number of tested hypotheses. Another probable cause of false positives may be population stratification and admixture (where populations with different rates of disease and possibly also with other genetic or non-genetic differences are unintentionally commingled in the same study).
Given the often modest effect sizes of common variants contributing to complex diseases, **false negative** findings may occur if the sample size is not sufficiently powered in the statistical sense to achieve even nominal significance ($p<0.05$). Of course, findings going in the opposite directions cannot account for false negative findings, but false negative findings can occur as a result of population admixture.
Finally, **true heterogeneity** between the populations of two different studies may exist. If a genetic variant's influence on disease susceptibility is only manifest on a certain genetic background (e.g. a particular susceptible gene variant on which the effect of the studied gene variant is affected by, is only occurring in one population) or environmental background (e.g. access to cheap palatable food), then genetic and environmental differences could account for a failure to replicate an initial reported association (Newton-Cheh and Hirschhorn, 2005).
Thus, as the inconsistencies may be due to false positive studies, false negative studies or true heterogeneity in association among different populations, meta-analyses can be used to assess small but real effects on common disease risk (Lohmueller *et al.*, 2003). However, it should be mentioned that publication bias (i.e. significant results, including the false positive studies, are more likely to be published than those studies finding no difference) does not *per se* affect the outcome of individual studies but may affect the outcome of meta-analyses of published studies (Easterbrook *et al.*, 1991).

1.4.4 Studies of variation in the *PPARγ2* promotor

As the studies of variation in *PPARγ* in general have shown quite inconsistent results, we (in paper 1) set out to evaluate whether other – and hitherto unidentified - variants in the *PPARγ2* promotor could be identified, and whether these variants, if existing, could explain the discrepancies in the results reported in previous studies (Larsen *et al.*, 2005b).

For the identification of novel variants, mutation analysis was performed on 61 obese T2D patients recruited from the outpatient clinic at Steno Diabetes Center. In addition, screening was also performed on 22 severely obese (mean BMI ~48 kg/m^2) subjects recruited from the ADIGEN cohort (Morberg *et al.*, 2003). Thus, we believe that we had a reasonable chance to detect genetic variants that could be observed at a higher frequency in patients with T2D or in severely obese patients compared to lean persons. As previous studies have identified the *PPARγ2* transcript as important in adipocyte differentiation (Tontonoz *et al.*, 1994;Saladin *et al.*, 1999;Zhang *et al.*, 2004), we chose to screen for variants in the promoter regulating the expression of *PPARγ2.* The examined region spanned a total of ~ 3400 base pairs, defined as nucleotide -3371 to +43 relative to the ATG translation initiation site. This region was split up in 7 segments, each ~600 bp in length. These segments were amplified using PCR, and via the denaturing high-performance liquid chromatography technique (dHPLC) (denaturation/renaturation of DNA double-helix allows for the detection of heterozygote carriers from homozygotic carriers of the particular allele with more than 95 % sensitivity) we identified a total of 8 gene variants, of which 7 had not been reported before. The subsequent identification of each of the nucleotide changes was done via direct sequencing of each of the 7 segments for those individuals that were initially identified as representatives of wild type or variant carriers via the dHPLC technique. The -1884T>A variant was only identified in one person and was not studied further.
Four of the identified variants (-816C>T, -1505G>A, -1881C>T and -2604T>C) were in complete linkage disequilibrium. The study indicates that the *PPARγ2* promoter region is highly polymorphic containing polymorphisms that are quite common (the allele frequencies of -792A>G, -882T>C, -2604T>C, -1953A>G were ~27, ~1, ~14 and ~11%, respectively), somewhat similar to the frequencies of the common well-studied Pro12Ala (~15%) and 1431C>T (~14%) variants.
For the association studies, a total of 6 variants, i.e. the -792A>G, -882T>C, -2604T>C, -1953A>G, Pro12Ala and 1431C>T (for Pro12Ala and 1431C>T some genotyping data was available from previous studies) which were only partially linked, were studied.
The study cohort included a sample of 234 obese and 323 non-obese subjects from the ADIGEN cohort. We examined the potential association between each of these variants with adult (age ~49 years) obesity, early-onset obesity (BMI>31 kg/m^2 at age ~20 years) or change in BMI during adulthood (from age 20 to 49) as well as their association with adult waist circumference, fat mass, lean tissue mass or body fat percentage. We did however not find any significant association to any of these measures.

1.4.4.1 Discussion of results

We studied a quite large proportion of what we considered to be the most important part of the *PPARγ2* promoter and found the region to be highly polymorphic.But if any of the identified variants has a large effect on the transcription rate of the *PPARγ2* gene, we would expect that this would have influenced the degree of adiposity in the subjects studied.
Therefore, as no association to any of the measures of adiposity was identified, we believe that in this population it is unlikely that any of these common promoter variants substantially affects the transcription of the gene – at least when each variant is considered on each own, i.e. without interaction with other gene variants or certain environments.
However, as we did only screen for variants in the *PPARγ2* promotor, we cannot state if variants in the coding region of *PPARγ2* (except for the Pro12Ala and 1431C>T variants) could have played a role in the development of obesity and T2D in the studied population. Also, this study does not rule out, that any of the identified gene variants could have an impact on the risk of T2D, if this increased risk is independent from the effects on obesity. Although our study population was well-characterized, as we had measures of BMI, waist circumference and body fat percentage measured by DXA-scanning, we are aware that our study also has its limitations. Most importantly, the sample size may not be large enough to detect more subtle effects on these measures. Considering only the Pro12 and Pro12Ala carriers, the sample size needed to attain a power of more than 0.80 to detect a difference in BMI of 1.0 kg/m^2, would be more than 2000 subjects (table 2a). However, in our dataset, the difference in BMI between these groups was actually much less (~0.2 kg/m^2 for the total

dataset of lean and obese subjects). Also, to detect a difference of 1% in body fat %, more than 3500 subjects are needed (table 2b). In our pooled sample of lean and obese subjects, the numerical difference in body fat % between the Pro12 and Pro12Ala group was ~0.5 % (Not significant, data not shown), and if this difference should have reached statistical significance, probably more than 15000 subjects would have been required (table 2c).

Table 2: Statistical power calculations

2a)	N	N	N	N	N		
Pro12	383	766	1149	**1532**	1915		
Pro12Ala	141	282	423	**564**	705		
Power	0.30	0.54	0.71	**0.83**	0.90		
Calculation of power for BMI, assuming difference of 1.0 kg/m^2 and SD/group = 7.0 (1 BMI unit ~ 3.19 kg BW)							
2b)	N	N	N	N	N	N	N
Pro12	374	748	1122	1496	1870	2244	**2618**
Pro12Ala	139	278	417	556	695	834	**973**
Power	0.20	0.35	0.49	0.61	0.71	0.78	**0.84**
Calculation of power for fat mass %, assuming difference of 1.0 % and SD/group = 9.0 (1 fat mass% ~ 0.96 kg)							
2c)	N	N	N	N			
Pro12	2690	5380	8070	**10760**			
Pro12Ala	1000	2000	3000	**4000**			
Power	0.32	0.56	0.74	**0.85**			
Calculation of power for fat mass %, assuming difference of 0.5 % and SD/group = 9.0 (0.5 fat mass% ~0.48 kg)							

N indicates number of subjects. Power calculations performed using the website: http://www.stat.uiowa.edu/~rlenth/Power/index.html / and the procedure: two-sample t-test (pooled or satterthwaite)

Thus, estimating the sample size needed is difficult and fully depends on the biological impact of each of these variants. Also, study population heterogeneity is a source of potential bias. In this study we selected the obese and control subjects based on their BMI (below or above 31 kg/m^2) at the mandatory draft board examinations which were carried out in the period between 1943 and 1977. This case-cohort sampling design may likely have introduced substantial genetic heterogeneity between the obese and the control subjects. In other words, there may be a significant accumulation of obesity-predisposing alleles in the obese cohort, and variants within the *PPARγ2* may actually only contribute very little to the overall sum of such predisposing alleles. However, although that we *per design* had created population heterogeneity, we do not believe that it invalidates neither the results nor the conclusions drawn from the study.

Although we failed to show any associations between any of the variants and the phenotypes studied, we cannot rule out that combinations of gene-variants could actually influence the transcription of *PPARγ2*, and thus the risk of obesity. Such effects could be evaluated using haplotype analyses. However, due to the relative small sample size and the relatively many gene variants identified, we believe that we cannot perform meaningful haplotype analyses in this study. If such analyses were to be performed, there would be a large risk that some of the haplotypes would be represented by very few study subjects, making the interpretation very uncertain. Also, the risk of false positive findings would increase due to multiple testing, and although corrections could be made, such additional testing should preferably be based on *a priori* established hypotheses (e.g. based on *in vitro* experiments) that the specific gene-variant combinations would have additional deleterious effects, as compared to single variants only.

Findings by Cole et al. have suggested that the PPARγ2 Pro12Ala polymorphism only accounts for approximately 1% of the variation in e.g. BMI and waist circumference (Cole *et al.*, 2000). If this is the case, significant effects on these measures may be quite unlikely to identify in small studies like ours, or in studies that are even minimally biased by heterogeneity – i.e. variations in other genes or

environmental factors contributing to these traits. These considerations were also pointed out by Altschuler et al. Having only a small sample size, one may fail to observe a small magnitude of effect. A meta-analysis of the association between the PPARγ Pro12Ala variant and T2D, including all reported association studies (including all published studies up to year 1999) identified a modest (approx. 1.25 fold) but significant increased risk of T2D in carriers of the Pro allele (Altshuler *et al.*, 2000;Lohmueller *et al.*, 2003). Thus, that study pointed out that not all studies found a significant association toT2D, but when all studies were analysed together, there was a small, but significant association.
But, as pointed out previously, meta-analyses also have their limitations, and also very large studies published after this meta-analysis have been quite inconsistent (Meirhaeghe and Amouyel, 2004). Moreover, no such similar meta-analyses have yet been reported for the association between the polymorphism Pro12Ala and obesity.
As it is our basic study premise that genetic variation will significantly affect gene transcription, splicing, mRNA stability, translation, or protein function, any variation with only marginal effects may not be identified. Therefore, the effect of the variants we identified could also be studied in functional *in vitro* studies. There are several drawbacks to *in vitro* studies, e.g. they cannot reveal the effects of heterozygous carriers and they cannot take into account any feed-back regulatory mechanisms that often operate *in vivo.* Therefore, although such data cannot be translated directly into effects observed *in vivo*, they can suggest which *in vivo* findings that may be expected.

Of course the phenotypes under study should be carefully chosen. As discussed in previous sections, variation of PPAR*γ* activity may not necessarily influence obesity as assessed by BMI, but the effects may be more pronounced on the distribution of fat within the whole body. In the clinical cases of lipodystrophy where mutations in *PPARγ* have been identified as the underlying cause, most *in vitro* studies have suggested that the variants induces a severely compromised PPARγ activity. Despite these findings, the body mass index has been reported to be normal in most cases of lipodystrophy, whereas only the fat distribution was abnormal (Hegele, 2005). Hence, one could argue that studying the effects on BMI of the Pro12Ala polymorphism or variants within the promoter region will not be useful if the real effects are changes in body composition, rather than just BMI.
Clinical evidence suggests that patients with T2D who are either Pro12 allele or Ala12 allele carriers, do not differ in their response rate to therapy with the PPARγ agonist pioglitazone, suggesting that the polymorphism does not have major effects on PPARγ activity (Bluher *et al.*, 2003).
It is very likely that most genetic variants will only have significant effects under certain environmental circumstances. In the heterozygous dominant negative *PPARg* hL466A knock-in mouse (PPARKI), which shows characteristics of hypoadiponectinemia, increased circulating levels of free fatty acids and hepatic steatosis (Freedman *et al.*, 2005), these effects were more pronounced when the mice were fed a high fat diet. Similarly, in humans the Pro12Ala variant and other variants that are expected to have only small effects on *PPARγ* expression or activity, such effects may only become apparent when influenced by an unhealthy diet and/or low physical activity. So far, only few studies of relative small sample sizes have been performed where also the interaction with non-genetic factors has been investigated.

1.4.4.2 *PPARγ* gene - environment interaction

As suggested by Becker, the impact of common disease-influencing alleles should be examined when they occur in different genetic backgrounds, in other genetic combinations, or influenced by different epigenetic or environmental factors (Becker, 2004). Such interactions have already been studied on the common Pro12Ala variant in *PPARγ* (discussed below).

Nutrient-gene interaction deals with the differential functional and eventual phenotypic effects of different doses of total energy intake or specific nutrients, in combination with various gene variants. Thus, nutrient-gene interaction can address inter-individual differences for the biological basis for development of obesity in some but not in other individuals eating the same diet.

Similarly, the environment-gene interaction addresses inter-individual differences for development of obesity when influenced by a particular environment.
Animal studies can be designed to model the effect of a specific genetic modification on susceptibility to the obesity-promoting effect of a high-fat diet and/or obesogenic environment, and the effect of these variables on gene expression in various tissues and organs. Nonetheless, only human studies will reveal the complex interaction between genetic, diet and environmental factors responsible for human obesity and hence for the current epidemic of obesity.

In a large sample involving more than 2141 subjects that served as a control group in the Nurses Health Study, there was no direct association between Pro12Ala genotype and BMI, but an association between total fat intake and BMI (higher fat intake associated with higher BMI) was found in Pro/Pro carriers, whereas no such association was found for the combined group of Pro/Ala and Ala/Ala carriers (Memisoglu *et al.*, 2003). A somewhat similar finding was reported from analyses of the Québec Family Study comprising a total of 720 adults. In Pro12Ala carriers, there was no association between total fat (and saturated fat) and components of MS, including BMI and waist circumference, whereas in the Pro12Pro carriers these associations were obvious. Overall though, the Ala carriers had a higher BMI and waist circumference (Robitaille *et al.*, 2003).

In the prospective Isle of Ely study, including about 600 non-diabetic subjects, Luan and colleagues found that among the Ala carriers there was an association between the ratio of polyunsaturated fat to saturated fat (P:S ratio) and BMI and fasting serum insulin level (higher P:S ratio associated with lower BMI and fasting insulin), whereas such an association was absent in the Pro/Pro carriers. Also, with a low P:S ratio the Ala carriers had a higher BMI than Pro/Pro carriers, whereas the opposite was seen in subjects with a high P:S ratio (Luan *et al.*, 2001). Overall, they did not find any association between Pro12Ala genotype and BMI or fasting serum insulin level.

Another study in the German Epic cohort comprising 154 subjects with BMI > 35 kg/m^2 and 154 age- and sex-matched controls demonstrated that Ala carriers had a higher odds ratio for obesity when having a high intake of arachidonic acid compared with subjects homozygous for the Pro allele (Nieters *et al.*, 2002).
Although not directly related to the development of obesity, in a small study of 56 Uruguayan Caucasians, Ala carriers seemed more susceptible to develop T2D, but there was evidence that this was caused by an excessive intake of trans-fatty acids, suggesting that the ala allele is more susceptible to the adverse effects of high intake of trans fats (Pisabarro *et al.*, 2004). Also, in 150 subjects participating in the KANWU study, Lindi and colleagues found that carriers of the Ala allele have a greater reduction in serum TG in response to 3 month fish oil supplementation as compared to subjects with the Pro12Pro genotype(Lindi *et al.*, 2003).

The possible interaction with physical activity has been investigated in 3 studies. In a group of 123 Japanese healthy men undergoing a physical activity program for 3 months, the improvements in fasting serum insulin and insulin sensitivity (HOMA-IR) were more pronounced in Ala carriers (Kahara *et al.*, 2003). In a cohort of 506 Caucasian men and women within the Ely Study, the findings were somewhat more complex; in Pro/Pro carriers the beneficial effect of an habitual high P:S ratio and a high physical activity (measured via heart rate monitoring for 4 days), the benefial effects of such habits correlated independently with fasting serum insulin levels (high P:S ratio and high physical activity were both associated with low insulin). Ala carriers did however, only have lowered fasting serum insulin levels, when both physical activity and P:S ratio was high, suggesting a multiplicative interaction (Franks *et al.*, 2004). In a Danish cohort of 29 healthy offspring of type-2 diabetic subjects, who underwent a training program for ten weeks, Ala carriers had a larger loss of body weight than Pro/Pro carriers, however they did not benefit further in terms of changes in VO_2max or insulin sensitivity (Ostergard *et al.*, 2005).

These studies emphasize the possible importance of interaction of this gene variant with inter-individual differences in nutritional and exercise habits, but the studies may also provide explanations of the discrepancies found in other association studies that neglect the role of the individual's everyday practices. On the other hand, the methodological issues pertaining to the search for obesity candidate genes are even more problematic in the study of e.g. nutrient-gene interaction. In the above-mentioned studies, estimates of habitual dietary intake and physical activity are often based on measurement methods with a considerable uncertainty compared to the more simple measurement of BMI.

1.4.4.3 Future association studies of *PPARγ* variants:

Due to the lack of convincing evidence of major important genes underlying the development of common obesity, there is still a need for large-scale and phenotypically well characterized cohorts (preferably using prospective designs).
It has been estimated that up to 75% of the publications on the association between risk of disease and particular gene variants are likely to be false positive ones. It has therefore been suggested that more stringent criteria are needed for improving the quality of future genetic studies to be able to better interpret such studies (Editor, 1999;Lohmueller *et al.*, 2003).
Such criteria seem also reasonable for the studies of the molecular genetics of obesity:
1) A single, nominally significant association should be viewed as tentative until it has been independently replicated at least once and preferably twice. A review of 25 association studies suggested that two studies with $P<0.01$ or a single study (other than the first positive) with $P<0.001$ is strongly predictive of future replication (Lohmueller *et al.*, 2003)
2) Large-scale studies should be encouraged, with collaborative efforts probably required to achieve the sample size of many thousands of case-control pairs,which is necessary for definitive studies of common variants with modest genetic effects. Even larger samples will be required to detect gene-gene or gene-environment interactions or associations specific to defined subgroups or to correct for testing association to multiple phenotypes.
3) To help increase the effective sample size, reports of association would ideally include a meta-analysis of all available published data to give a more robust estimate of the genetic effect. To facilitate such meta-analyses and minimize publication bias, all disease association studies that meet minimal quality standards should be published. Such standards could include explicit phenotype definitions, complete listing of all phenotypes analyzed, precise localization of the polymorphism(s), low genotyping error rate, analysis that avoids overlap with previous studies and availability of genotype counts for cases and control subjects (or equivalent data for family-based studies).
4) As many studies will not be able to fulfil such high standards and because of risk of publication bias, such studies could be published (positive as well as negative findings) in widely accepted and curated web sites and/or in brief "negative or suggestive results" sections of specialty journals with sufficient credit to provide incentives for publication (Hirschhorn *et al.*, 2002). Also, as these common variants often only have modest effects, the impact of each variant should be considered (e.g. also reported) together with other risk factors associated with obesity development (i.e. environmental factors including physical activity, diet patterns, behavioural and socio-economic factors) (Ardlie *et al.*, 2002). Although such a further thorough characterization of the study population is difficult and often not possible, there is increased understanding that further insight into gene-gene and gene-environment interaction is needed, and such studies are indeed on the rise. Preferably large-scale prospective intervention studies with randomised designs should be employed, as such studies will be able to include thorough characterizations of the study population including also the registration and controlled manipulation of several environmental variables, while eliminating the risk of population stratification.

For the study of *PPARγ* variants, there may be some more specific study designs that could be considered, taking into account the knowledge about its possible role in lipid and carbohydrate regulation:

In addition to the phenotypes considered in paper 1 (Larsen *et al.*, 2005b), other and more novel phenotypes could be studied, such as abdominal fat for given body fat percentage (preferably assessed by MR or CT-scan), ectopic fat depots and waist/BMI ratio. As gene effects may only become visible when the subjects are studied under particular conditions or challenges, glucose tolerance test or meal tolerance test (e.g. FFA suppression) could be considered. In addition, gene expression of *PPARγ2* and expression of genes regulated by PPARγ in adipocytes isolated from fat biopsies from various body compartments would be of major interest.
Finally, as previously mentioned, haplotype analyses within the *PPARγ* locus could be considered if the sample size is large enough. As illustrated in appendix A + B, the *PPARγ* locus possess several SNPs, with linkage disequilibrium values (R^2) up to 1 within the nearest 20 kb, documenting the large genetic variability and variation in linkage within this region in this population of European American descent.

1.4.5. In summary: The role of polymorphisms in *PPARγ*2 in the pathogenesis of obesity, T2D and CVD

There is both theoretical and clinical evidence that *PPARγ* is a plausible candidate gene involved in the molecular pathogenesis of obesity. The evidence that genetic variation within the *PPARγ* locus is implicated in common obesity is however still limited. Clearly the evidence for involvement of common polymorphisms (e.g. the Pro12Ala) in common obesity is highly controversial, and the evidence from *in vitro* studies, as well as from transgenic animal models and *in vivo* measures in humans does not always fit with the findings from genetic association studies, questioning the validity of the studies available. Nonetheless as discussed, there may be several explanations for the observed discrepancies, and more – and preferably more optimally designed - studies are needed to clarify these inconsistencies.
Ideally, to study such effects of each of the identified variants within *PPARγ* would require a study design that would mimic "life-long" situations, taking into account also the influence of different environmental factors. Such a study is unfortunately quite unfeasible in humans. Probably the best study that one could imagine to establish proof of concept would be to study transgenic e.g. Pro12Ala mice in life-long studies while also controlling the amount of physical activity and the diet composition. In conclusion, we undertook a thorough analysis of the variation in the *PPARγ2* promoter, in order to assess whether hitherto unknown genetic variants could explain the inconsistent findings between *PPARγ* variants and obesity. It was, however, not possible to offer such explanations.

1.5 Conjugated Linoleic Acid – a natural and novel approach in the treatment of obesity?

We have recently conducted a review addressing the effect of Conjugated Linoleic Acid (CLA) on body weight, body fat content and various health outcomes in the literature of human and animal studies (Larsen *et al.*, 2003a). Subsequently we have performed an intervention trial studying the effects of supplying obese humans with CLA in addition to their usual diet after an initial weight loss (Larsen TM *et al.*, 2005).

In the following I have addressed the biological effects of different types of fatty acids, and in particular the effects of CLA, with regard to their effect on body weight and body fat.

The effect of CLA on fat cells as well as the data from cellular, animal, and human clinical studies is reviewed to further understand if and how long-term CLA treatment in humans may affect obesity and risk of T2D and CVD. Also, any potential safety issues associated with CLA supplementation are discussed.

1.5.1 The definition of CLA

As described in paper 4 (Larsen *et al.*, 2003a), CLA is a collective term used to describe the mixture of positional and geometric isomers of linoleic acid (c9,c12-C18:2) with conjugated double bonds (i.e., the two double bonds are separated by one single bond). These double bonds, each of which may be in the *cis* or *trans* configuration, can be in any position on the carbon chain. They are, however, most frequently found in positions 8 and 10, 9 and 11, 10 and 12 or 11 and 13, respectively. CLA is sold commercially as diet supplements. The CLA in commercial supplements is obtained by alkaline isomerization of vegetable oil enriched with linoleic acid (e.g. from safflower oil or sunflower oil). The fatty acid composition in the most sold CLA-preparations is dominated by 2 main isomers, e.g. the 9c,11t-isomer and 10t,12c-isomer (see Figure 7), including in some preparations, a minor content of other isomers of conjugated linoleic acid (Figure 8). The 9c,11t-isomer is the main CLA occurring naturally in foodstuffs (up to ~80% of total isomers). Since most of the CLA intake and therefore the 9c,11t-isomer arise from ruminant products (dairy and meat), this isomer is called rumenic acid. It is a byproduct of microbial biohydrogenation that takes place in the rumen from linoleic acid (and α-linolenic acid) occurring from plants and ingested by ruminants (Appendix D). When dealing with the bioactivity of CLA, it is likely that their structural peculiarities underlie some of their radically different actions when compared to linoleic acid. Nevertheless, only few studies have addressed the discrete potency of each of these isomers, or the particular synergistic or competitive effect of having several isomers present together in the same mixture (Larsen *et al.*, 2003a). In comparison, the most common industrially produced trans fatty acids (TFA) (which are usually trans isomers of oleic acid c9-C18:1) is elaidic acid, t9-C18:1, vaccenic acid t11-C18:1 and other (yet un-named) fatty acids with different positions of the trans binding, illustrated in Appendix F (Steen Stender and Jørn Dyerberg, 2003). For a list of common fatty acids, please see Appendix C adapted from Shields et al.(Shields et al., 1999).

Figure 7: Structure of linoleic acid and the most common CLA-isomers c9,t11 (9Z11E) and t10,c12 (10E12Z)

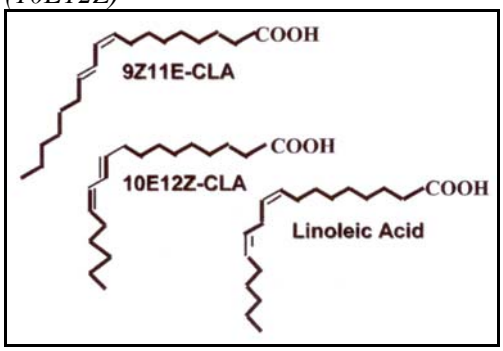
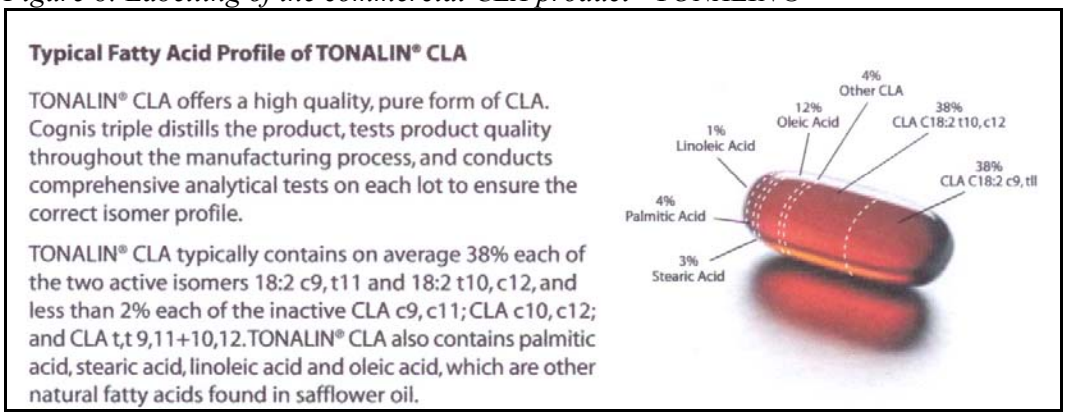


Figure 8: Labelling of the commercial CLA product "TONALIN®"



Adapted from www.cognis.com, August 2003

1.5.2 The molecular effects of CLA

As discussed in paper 4 (Larsen *et al.*, 2003a), there is substantial evidence that CLA attenuates adipocyte differentiation in various cell systems. Besides, most studies find that this effect can be ascribed solely to the t10,c12 isomer, as it has been shown that the t10,c12 isomer lowers TG accumulation in preadipocytes, whereas the c9,t11 isomer tends to increase TG content. In addition, there is evidence, that this effect is caused, at least in part, by a downregulation of the expression (and possibly inactivation) of the transcription factor PPARγ, which is known to be a strong inducer of adipogenesis. Recent studies in both mouse 3T3-L1 adipocyte cells and human SGBS cells have found that the t10,c12-induced attenuation of adipocyte differentiation is dependent on both time point and duration of treatment, i.e. that early and prolonged treatment substantially decreases the expression of lipogenic marker genes (*PPARγ*, *C/EBPα*, *LXRα*, *aP2* and *CD36*) and severely down-regulates the lipid accumulation in mature adipocytes (Angeles ZM *et al.*, 2004;Granlund *et al.*, 2005b). In addition, a recent study suggests that small structural changes (i.e. changing to trans/trans binding or introducing an α-methyl group in the molecules tested; c9,t11, t10,c12, TTA, EPA or DHA) totally abolished their "native" effects, i.e. for the t10,c12-isomer, its down-regulation of adipogenic markers, including PPARγ, returned to levels of control treatment if it had an α-methyl group added. Thus, the "native" isomer t10,c12 is highly specific in preventing lipid accumulation in adipocytes (Granlund *et al.*, 2005a).

To assess the binding affinity of different fatty acids on the PPARγ transcription factor, competitive assays against the radioactively labelled highly selective PPARγ ligand, thiazoledinedione BRL49653 (Avandia, rosiglitazone) have been performed. There it was shown that saturated fatty acids in general had a lower affinity than polyunsaturated and monounsaturated fatty acids (Xu *et al.*, 1999;Desvergne and Wahli, 1999). The degree of affinity can however, not necessarily be translated into degree of agonist activity. Still, using a co-activator-dependent receptor ligand assay (CARLA) (i.e. also taking into account the important interaction with the associated transcription complexes

controlling transcription), similar results were obtained (Krey *et al.*, 1997). But in co-transfection studies, the potency of saturated fats were not always lower than for polyunsaturated and monounsaturated fatty acids (Kliewer *et al.*, 1997). Overall though, the PPARγ receptor is considered a low-affinity receptor, using a very broad range of different fatty acids or fatty acid derivatives as low-affinity ligands. Some data indicate that some fatty acids do have a higher PPARγ activating capacity than others. Thus, 15-deoxy-$\Delta^{12,14}$-Prostaglandin J2 has a higher affinity than other fatty acids(Forman *et al.*, 1995). Also, nitrolinoleic acid (LNO_2) which is commonly found in human blood at concentrations of ~500 nM has been shown to activate PPARγ as much as the highly specific TZD drugs (Schopfer *et al.*, 2005).
Although the *in vivo* effects of each particular fatty acid isomer can be predicted from *in vitro* studies, the actual concentrations of the fatty acids may in some situations not be linearly related to the dosing regimens if there is a high metabolism and turn-over of the fatty acids, decreasing the concentration of the particular isomers in the blood and in various types of body tissues.

1.5.3 Effects of CLA in animal studies

As discussed in paper 4 (Larsen *et al.*, 2003a), numerous studies have investigated the effect of CLA on different animal models, and under a variety of different feeding conditions. In animals, it is widely recognized that feeding CLA (various mixtures of isomers) to different animal species results in changes in body composition, i.e. lowering of body weight in some cases, and a relative decrease in body fat mass together with relative gain of lean body tissue in most studies. The effects in animals are highly variable, possibly depending on a variety of factors including, but not limited to, species, age, gender, dosage, concomitant feeding regimen (e.g. total fat content of diet), and duration of CLA feeding, and certainly also the CLA isomer tested. Furthermore, adult animals may not be as susceptible to these effects – at least in rats, as it has been shown that adult rats (aged >13 weeks old) were not affected by 6 weeks of 1% CLA supplement (Mirand *et al.*, 2004). Although not yet definite, most studies have identified the t10,c12 isomer, rather than the c9,t11 isomer, as being responsible for the attenuation of body weight and body fat gain. Most studies in animals have evaluated the effects on body weight and body composition, but some of the studies may also give hints on the effect of CLA-supplementation and its effects on risk for T2D and CVD, although one has to be very cautious to extrapolate results from animal studies into potential use in humans. Unfortunately most evidence supports the notion that feeding CLA may adversely affect liver metabolism and have negative effects on glucose homeostasis in mice. Also it is likely that the major part of these adverse effects can be ascribed to the t10,c12 isomer. Moreover, the t10,c12 isomer seems to be responsible for the so-called “milk fat depression syndrome” (i.e. a reduced fat content in the milk) observed in cows (Larsen *et al.*, 2003a).

Recent data support the findings from the studies in cell systems. In mice fed a mixture of 1% CLA for 28 days, the expression of *PPARγ*, *aP2* and *adiponectin* in adipose tissue decreased as early as 2 days after dosing initiation. Subsequently, the amount of adipose tissue mass was attenuated, and the fasting serum insulin levels increased dramatically, which was explained by pancreatic beta cell hyperplasia. In the liver, the expression of *PPARγ* and *aP2* increased dramatically, likely explaining the observed increase in hepatic lipid content (Poirier *et al.*, 2005a;Poirier *et al.*, 2005b). Subsequent studies have observed that these effects are reversible, when the CLA is removed from the diet (Martin J-C *et al.*, 2004). Similar findings were reported by another research group. In this case, mice had decreased fat mass and developed hepatomegaly when fed CLA. Furthermore, addition of increasing amounts of fish oils to the diet, substantially reversed these effects of CLA, increasing body fat and lowering hepatic TG content (Ide, 2005). Apolipoprotein E knockout mice are sensitive to the development of artherosclerosis due to a deficiency of LDL-cholesterol clearance in the liver. Also in these mice, t10,c12 has been shown to increase insulin resistance and to increase lesion area in the heart, suggesting a progression of inflammation and atherosclerosis (de Roos *et al.*, 2005a;de Roos *et al.*, 2005b).
Moreover, in contrast to CLA, apart from being negative regulators of hepatic lipogenesis and activators of hepatic PPARα, long-chain n-3 polyunsaturated fatty acids may also attenuate

hyperinsulinaemia and hyperglycemia (Sekiya *et al.*, 2003). Somewhat in contrast, and therefore slightly surprising, another study in mice supplied with (only) 0.2 % t10,c12 CLA for 4 weeks did not find any down-regulation of the expression of either *PPARγ* or *aP2* in adipose tissue, although body fat gain was reduced (Kang *et al.*, 2004).

There are indeed suggestions that CLA may have biological similarities to other dietary trans fats. From a structural viewpoint, the CLA isomers should be considered trans fats. Overall there is substantial evidence that CLA or other trans fats are likely to compete with the metabolism of essential fatty acids in a variety of different biochemical pathways, which could give deficiencies related to the lack of important cellular lipid components that should be produced from dietary intake of essential fatty acids (Kinsella *et al.*, 1981;Hill *et al.*, 1982). Appendix E shows the current evidence of – at least part of - the metabolic fate of the 2 isomers c9,t11 and t10,c12 CLA (Martin and Valeille, 2002).
Such effects on lipid metabolism does not necessarily decrease fat gain. But, if CLA does attenuate fat gain in animals, so could other trans fatty acids also do, and vice versa.
Thus, studies of trans fats in animal models and their impact on body weight change etc. might give some hints about the general effects of CLA also. Unfortunately, there are only few such studies available. As early as in 1979, Hwang and Kinsella studied the effects of trans fat on growth rate in rats and found that trans C18:2 (n-9,12) severely attenuated growth rate compared to either c9,c12-C18:2, to a 50% mixture of each of the cis,cis and trans,trans isomers or to hydrogenated coconut oil (Hwang and Kinsella, 1979). More recently, Colandré and colleagues studied the effect of 30 days feeding with trans C18:1 (n-9) fats in rats, and did not find any effects on body weight gain compared to c9,c12-C18:2 or 18:0 fats, although the trans fats seemed to increase hepatic TG content (Colandre *et al.*, 2003). Partially hydrogenated fish oils have also been shown to increase LDL-cholesterol and LDL/HDL ratio in rats (Morgado *et al.*, 1999).
Also, trans fat has been shown to decrease insulin sensitivity to a greater extent than saturated fats, possibly via a downregulation of PPARγ and LPL in epididymal fat tissue (Saravanan *et al.*, 2005). Studies of isolated mouse islets have also shown that the trans isomers, trans vaccenic acid and elaidic acid elicit a higher maximal insulin output that the respective cis isomers (Alstrup *et al.*, 1999).

1.5.4 Effects of CLA in human studies

How to assess effects on body weight and body composition
As discussed in paper 2 (Larsen TM *et al.*, 2005) and paper 4 (Larsen *et al.*, 2003a), quite a few studies on CLA have now been carried out in humans. Although the studies identified in paper 4 may have looked for a variety of different outcomes, we will focus on the effects reported for changes in body weight and body composition and on changes in risk factors of T2D and CVD.

To study such effects it is necessary to use reliable measurement methods. Regarding the effects on body composition, the effect variables most often studied are changes in body weight, waist and hip circumference, whereas body composition is less often studied, possibly because measures such as fat percentage, fat mass or fat free mass can only be assessed in a precisely fashion if appropriate equipment (e.g. DXA-scanning) is available. Similarly, the assessment of changes in abdominal and/or visceral fat depots requires access to CT or MRI-scanning methods. Overall, as discussed in the review paper (Larsen *et al.*, 2003a) we did not find any convincing data supporting that CLA has effects on body weight, and there was no unequivocal evidence of an effect on body fat percentage, although not all studies had used optimal methods to measure such changes in body composition. Also, we concluded that most studies actually suggest that CLA may actually induce adverse effects, including insulin resistance in pre-diabetic patients.

How to predict risk of T2D and CVD

As described in the section about the definitions of the metabolic syndrome, there are quite a few different variables that are often used to assess changes in risk of T2D or CVD. In relation to T2D, fasting blood levels of glucose and HbA1c are often used. Also indices of insulin sensitivity or glucose tolerance can be evaluated using fasting serum specific insulin, HOMA-IR, clamp-techniques and oral glucose tolerance test. There are quite a lot of different blood component that by them selves or in combination are associated with future risk of cardiovascular disease. For clinical practice, the most used are blood levels of total cholesterol, LDL, HDL and TG, whereas assessment of artery intima-media thickness (IMT) is more labour-intensive and therefore less used.

Newer markers of increased risk of CVD include measures such as blood content of hsCRP, adiponectin, fibrinogen, coagulation factor VII or PAI-1. In addition, a list of emerging markers includes, but are not limited to; von Willebrand factor, MCP-1, MMP-9, sCD40CL, ICAM, VCAM, VEGF, endothelin, E-selectin, BNP, ApoB, Lp(a), TNFα and IL-6.

Most of these measures have by themselves or in combination been associated with future risk of development of cardiovascular disease. But there may be a substantial degree of complexity in the use of these novel markers. Thus, although changes in behaviour or medical treatment known to decrease future risk of cardiovascular events (e.g. increased physical activity, weight loss or statin treatment), would be expected to be associated with beneficial changes in these risk factors, this is not always the case. Often, some risk markers may be improved, whereas others are unaffected or may even be adversely affected. Such apparent discrepancies may be caused by measurement error or measurement variability, but such effects may also be real.

1.5.4.1 Does CLA resemble trans fats? – evidence from effects on risk of T2D and CVD.

As it has been shown many years ago (Mensink and Katan, 1992) and recently reviewed by Ronald P. Mensink (Mensink, 2005), different fatty acids (e.g. n-3 fats versus saturated fats) do have different effects on serum lipoproteins, and also isomeric fatty acids, either different in the direction of the double bond (e.g. c11-C18:1 vaccenic acid versus t11-C18:1 vaccenic acid) or in the position (e.g. c9-C18:1, oleic acid versus c7-C18:1 un-named) do exhibit different effects.

Concurrently there is quite clear evidence that trans monounsaturated fatty acids (e.g. t9-C18:1, elaidic acid) have adverse effects on the serum lipoprotein profile, as it increases serum LDL cholesterol and lowers serum HDL cholesterol levels when compared to e.g. oleic acid (c9-C18:1)(Mensink *et al.*, 2003). Trans fatty acids are also found to increase serum levels of apolipoprotein(a)(Mensink *et al.*, 1992;Nestel *et al.*, 1992;Mensink *et al.*, 2003;Gatto *et al.*, 2003)

These changes in serum lipoproteins induced by TFA would be expected to increase the risk of coronary heart disease. A meta-analysis of randomised studies estimated that the effect on a 2 E% increase in TFA would influence lipoproteins to an extent, corresponding to an increased risk of 5% for developing coronary heart disease (Ascherio *et al.*, 1999). In comparison, the average intake of TFA in Europe in the years 1995-1996 was 0.87 and 0.95 E% in men and women respectively, an amount similar to the intake in Denmark (van Poppel, 1998;van de Vijver *et al.*, 2000). Also, several of the largest epidemiological prospective studies, i.e. Nurses Healthy Study, Health Professionals Follow-up study, The Alpha-Tocopherol Beta-Carotene Cancer Prevention Study and the Zupthen Elderly Study have demonstrated that trans fat intake is associated with increased risk of coronary heart disease: The increased risk of coronary heart disease for an increase of 2% of energy in TFA was estimated to be approx 25% in a pooled analysis of these 4 studies (Oomen *et al.*, 2001).

The discrepancy for these estimates (5% based on lipoprotein profiles vs. 25% based on epidemiological studies) is yet unclear, but may be ascribed to the use of different study designs or it may be caused by adverse effects of the TFA on CVD risk that are mediated by other factors that act independently of changes in lipoproteins. In this context, studies have also shown that TFA impairs endothelial function (de Roos *et al.*, 2001), increases the level of pro-inflammatory risk markers such as hsCRP and E-selectin(Baer *et al.*, 2004). Further, there are suggestive evidence of an increased heart rate after a high TFA intake (Dyerberg *et al.*, 2004).

The molecular mechanisms by which TFA may infer increased risk of CHD is more or less unknown. There are suggestions that TFA via its incorporation into cardiac cell membranes may

modulate membrane ion channel function and thereby have arrhythmogenic effects (Katz, 2002); however the relative importance of TFA´s possible adverse effects on artherosclerosis progression, function of the arterial wall and cardiac rhythm is largely unknown. Hence, TFA may give increased risk of peripheral atherosclerosis, heart disease and possibly stroke, but the molecular pathogenic mechanism may be different for each of these diseases.
Similarly, TFA may increase the risk of T2D via several mechanisms which could include, but are not limited to, increased insulin resistance in either muscle, liver or via effects on adipose tissue.
In humans, studies of relative short duration have been performed. A 4 week cross-over study of 14 healthy women found an increase in serum levels of total cholesterol/HDL, triglycerides and apoB, but no effect on insulin sensitivity during intake of 5.1 E% TFA (Louheranta *et al.*, 1999), whereas a 6 week cross-over study in 16 obese type-2 diabetic patients found no effects on fasting lipids, glucose and insulin, but TFA increased postprandial insulinemia, similar to a diet high in saturated fats (Christiansen *et al.*, 1997).

Thus, it is of major interest to study whether conjugated linoleic acid that are currently available in the diet (primarily c9,t11 linoleic acid) or as dietary supplements (usually c9,t11 and t10,c12) have effects on lipoproteins, plasma markers of low-grade chronic inflammation or on IMT.
A meta-analysis of more than 11.000 subjects involved in 37 intervention studies (Yu-Poth *et al.*, 1999) has reported that certain beneficial changes in plasma lipids (i.e. LDL- and HDL-cholesterol and TG) can be expected for every 1-kg decrease in body weight. Therefore, if CLA introduces weight or fat loss, it is of interest to show if these effects are followed by improvements in the lipoprotein profile, or if CLA has some other – yet unidentified – adverse effect on lipoprotein profile, that offsets the expected changes seen with weight loss.

1.5.4.2 Human studies of CLA – effects on body weight and risk of T2D and CVD.
As discussed in paper 4 (Larsen *et al.*, 2003a), the CLA-isomers commercially available in dietary supplements are usually a 50:50 ratio of c9,t11 and t10,c12. However, as also described, the actual amount and composition of the CLA supplements used in the various studies may differ significantly and therefore the results are not always directly comparable. In general, although there are quite a few studies of CLA, also in humans, most are of quite short duration (<6 months) and many of the studies are of a poor quality (lack of placebo-group, unreliable measurement methods etc.).
Most animal studies have examined the effect of CLA during growth. This is a state where the adipocytes are actively differentiating and therefore the anti-adipogenic effect of CLA may in such circumstances be more pronounced compared to situations of weight stabilisation. Indeed, a study in rats have suggested that the anti-adipogenic effect of CLA may not be very effective in adult rats. Also, in humans, most studies of CLA have been performed in weight stabile lean subjects.
In the CLA005 (Paper 2) (Larsen TM *et al.*, 2005), we tested the hypothesis that in obese subjects, after an initial large weight loss, CLA would help to maintain this initial loss of body weight and body fat to a larger extent than placebo.

In the CLA005 study (Paper 2) (Larsen TM *et al.*, 2005) we assessed the effect of a mixture of c9,t11 and t10,c12 CLA isomers on 1-year body weight regain in obese subjects. Data on body weight and body composition were available for a total of 83 participants (40 in CLA group and 43 in placebo group) who completed 25 and 52 weeks of treatment (Intention-To-Treat data which were defined as those who completed 25 weeks of treatment were carried forward to week 52 for 6 subjects). We assessed changes in total body weight, body composition (using DXA-scanning), and we obtained measures of waist and hip circumference, analyses of fasting circulating levels of glucose, insulin, growth hormone, IGF-1, total testosterone, TSH and various safety analyses including leucocyte count, as well as energy-intake via 3 day self-recorded diet registrations at baseline and after 25 and 52 weeks treatment, respectively.
Overall, we found no evidence that CLA effected changes in body composition, blood glucose or insulin, but we did not assess changes in lipoproteins. Taken together, this finding is in agreement with most of the previous studies with a duration of ≤ 6 month, including a recent 6 month study of

approx. 50 subjects (Tricon *et al.*, 2004). However, our findings stand in contrast to the only other studies with a duration of >6 month (Gaullier *et al.*, 2004;Gaullier *et al.*, 2005). As pointed out in paper 2, the reason for these differences may be due to the different study design, e.g. in our study supplementation was given during a period of weight regain.

Regarding the effects on cardiovascular risk factors, Tricon et al. have recently reviewed the effect of CLA on plasma lipoproteins, and although there is a substantial degree of variation in the results obtained in different studies, the overall findings suggest, that if CLA has any effect – it seems to decrease the serum HDL-cholesterol level and to increase the ratio of LDL/HDL cholesterol (Tricon *et al.*, 2005). A recent cross-over study assessed the effect of butter containing naturally occurring CLA (i.e. c9,t11-isomer) in 16 overweight or obese men and found a slight increase in total cholesterol/HDL-cholesterol compared to butter without CLA (Desroches *et al.*, 2005). Therefore, it may be premature to provide a solid answer. With respect to other risk factors of T2D and CVD, such as hsCRP, adiponectin or Lp(a), so far only few studies of limited duration are available.

In our study we found no clear evidence of changes in insulin sensitivity as measured by the HOMA-IR index, nor did we identify effects on the various blood hormones assessed. However, we found that CLA increased blood leucocyte count. This has also been found in several previous studies (Gaullier *et al.*, 2004;Gaullier *et al.*, 2005), indicating that this is a robust finding.
This finding may be of some concern, since there is accumulating evidence that low- to moderate grade inflammation is associated with, and possibly also involved in the pathogenesis of atherosclerosis (Hansson, 2005). Hence, this finding may indicate that CLA could increase the risk of atherosclerosis via increased inflammation, an effect that may be independent of any effects on changes in body composition, insulin sensitivity or lipid profile.
Though, as discussed in paper 2, the clinical relevance of this finding is unclear, it adds further evidence that these fatty acids possess unique characteristics that should be investigated further.

There are several shortcomings in the study presented in paper 2 (Larsen TM *et al.*, 2005). We did only assess compliance to the treatment via counting of non-consumed capsules returned to the study personnel. Clearly, a more objective measure of drug compliance could have been assessed via analyses of e.g. fatty acid analyses of blood platelets. Also, we did not evaluate treatment effects related to risk of cardiovascular disease. Changes in blood lipids could have been analyzed to assess the changes in risk of CVD, plasma adiponectin could have been analyzed to assess changes in insulin sensitivity, and the inflammatory risk marker hsCRP could also have been a useful indicator of cardiovascular health. However, the primary aim of the study was to assess the effect on changes in body composition and several safety factors. This aim was satisfactorily answered.

1.5.5 In summary: Is CLA a trans fat or a healthy fat?

The totality of evidence suggests that CLA, or more specifically the t10,c12-isomer, has various effects on lipid metabolism, including effects on adipocytes and hepatocytes. However, even though its possible effects on body fat accumulation seems a tempting modality to treat obesity, there is now a substantial amount of data indicating that it is followed by metabolic effects on adipocytes and hepatocytes, which seem to lead to a modest increase in the development of insulin resistance, T2D and possibly CVD. Although data is still sparse, it seems that at least some of the effects of CLA (or perhaps specifically the t10,c12 isomer) are similar to the biological effects obtained from studies of trans fatty acids, including effects on transcriptional regulation in adipocytes, effects on blood cholesterol levels and animal growth. Thus, although CLA currently may be one of the best studied dietary supplements, both in terms of efficacy and safety, it is unfortunately far from certain that CLA has a potential benefit to human health. If CLA is compared to the current medications Xenical (orlistat) and Meridia (sibutramine), (or Rimonabant coming up), that are approved and used for weight loss, there may be some studies suggesting that CLA on a long-term basis has some effects on fat loss, however it does still not seem as effective as Xenical and Meridia to decrease body fat loss. Also, our own study (Larsen TM *et al.*, 2005) does not support that CLA is effective, at least not

during weight gain in obese persons. Also, in terms of changes in risk factors of development of MS, T2D or CVD such as levels of glucose, insulin, blood pressure, cholesterol, hsCRP or others, it is of concern that CLA has not shown any significant improvements in these traditional risk factors. Therefore, based on the current evidence, the (if any) potential use of CLA seems to be restricted to a cosmetic purpose only (i.e. to obtain a slight decrease in body fat) - and not for health benefits. For many health professionals, the missing link between the loss of fat mass (which is observed in some studies) and changes in these disease risk factors is actually a major concern, and it also gives some scepticism about the overall health effects of CLA which therefore needs further research.
Also, the get a regulatory approval as a weight control drug, the US Federal Drug Administration specifically states that "Measurement of obesity-associated cardiovascular risk factors (lipids, blood pressure and glucose tolerance) during drug administration is encouraged, as they may have a place in determining the balance of benefit vs. risk for the drug. If one or more of these factors deteriorates or is not improved, the risk associated with this deviation must be considered in making a benefit-to-risk decision for the drug" (FDA, 1996)".

2. Perspectives and future research

PPARγ:

Surely, for single gene disorders, molecular genetics have helped to determine cause, prognosis, and appropriate treatment, but for more common polygenic diseases such as T2D, the role of molecular genetics will more likely – at least in the short term - be to enhance understanding of disease pathogenesis and help formulate preventative and treatment strategies. While genetics undoubtedly play a role in the development of many metabolic and vascular conditions, the prevalence of obesity and T2D has increased too rapidly over the last two decades to be the result of alterations in the gene pool. The obesity epidemic is more likely to be explained by an interaction between genotype and acquired factors. Currently most evidence supports the notion that the sedentary lifestyle and high-calorie diet common to Western societies is the major contributor to the obesity pandemic – a key risk factor for MS, T2D and CVD in genetically susceptible individuals.

The current evidence suggests that the common variations within *PPARγ* have only a marginal effect on the risk of obesity and T2D. Therefore, as with many of the other so-called "common variant – modest effect" genetic variants, the study of *PPARγ* variants needs to be further examined using larger well-characterized and statistically powered study samples, preferably also assessing gene-gene and gene-environment interaction effects. It would also be useful to do comparative analyses of genetic versus environmental (unhealthy diet, physical activity, socio-economic status) obesity-contributing effects within the same study sample, in order to do direct comparisons of the importance of each of these variables. Furthermore, novel phenotypes could be investigated, such as the ratio between waist circumference/BMI as suggested by Bigaard et al. (Bigaard *et al.*, 2003), different physiological "challenges" (e.g. meal test) or the impact on early-onset disease. Mouse models with increased expression of the PPARγ protein have not yet been reported, but such a model could maybe help to clarify if more subtle variations in PPARγ protein levels (i.e. mimicking the effects of *PPARγ2* promotor variation) would have clinically relevant effects, which therefore could help to sort out the reasons for the discrepancies reported in the many human association studies. Only if such data is available and if most findings are corroborative, genetic testing could be used in the clinic for diagnostic and treatment purposes. However, currently the available data for the importance of single-gene effect on the molecular genetic pathogenesis of common obesity is not convincing.

It may be argued that genetic influence may have minimal effects on the development of obesity, and that environmental changes may be the overall responsible for the obesity epidemic in urbanized civilizations. If that is the case, then it is argued that subtle effects from single gene variations would be unlikely or merely impossible to identify. However, with many novel genetic methods currently in development, including the availability of genome-wide SNP assays with the potential to look at the effect of thousands of different gene variants at a time, it may be that future efforts will be much more effective in identifying important genetic contributors of common obesity. These efforts will however, still rely on the premise that the contribution from genetic variation is quite large, and that it has an impact that is as a minimum as large, and preferably larger than the inherent inter-individual "noise" or variability from other non-measured genetic and non-genetic factors that may remain in the study sample.

CLA:

Is seems plausible that different types of fatty acids have different biological effects. We have solid evidence about the effects of the most common fatty acids, including fatty acids derived from dairy, meat, vegetable and fish oils, and we also have some knowledge about the effect of eating products high in - in most cases unspecified types of – trans fats. But the precise biological activity from each particular trans fatty acid is far less investigated.

Hence, from various mechanism studies and intervention studies there is robust evidence that TFA induces adverse effects on cardiovascular risk factors (i.e. LDL and HDL), and from epidemiological studies we have evidence that TFA increases the risk of cardiovascular events and for the development of T2D. However, epidemiological studies cannot establish a cause-and-effect

relationship without support from other types of studies. Furthermore, the assessment of diet consumption is quite inaccurate, and may be very much affected by non-assessed factors which could introduce bias. Clearly there is a need to study further the effects of single isomers of fatty acids, and preferably to perform such studies as comparative dose-rising studies in order to evaluate if some effects are only apparent with low- or high concentrations. As listed in appendix C (which is certainly not exhaustive), there is a huge variation in the structural composition in the fatty acids that we consume in our diet, and each of which may have different metabolic effects.

Regarding CLA, there are also some obvious highly controversial findings in the existing literature: For instance Vessby and colleagues have provided evidence that when given alone, the CLA isomer c9,t11 and the t10,c12 isomer increase insulin resistance, whereas there was no effect of a mixture of these isomers. This may apparently seem quite peculiar and if it proves to be true, it will be very interesting to look further into the mechanisms behind this observation. Such interactions will certainly also suggest that studies of single isomers cannot be used reliably to illustrate the effects on real life situations, where different fats are consumed together and in various amounts.

Many fatty acids have a natural origin, but also many foods (and dietary supplements such as CLA) nowadays may contain a wide range of different fatty acids that cannot be found at all, or at least only in small amounts in natural foods. It can be argued that our body metabolism may not be genetically adapted to (or at least not optimally adapted) such "odd" fatty acids in excessive amounts, and that these fatty acids therefore may have deleterious effects in the body.
It is plausible that such "odd" fatty acids not only have adverse effects in single tissues, but may have biological effects in all biological pathways, in a wide variety of tissues and organs where it competes with other more natural fatty acids. Despite the evidence that CLA may have characteristics of other trans fats, some of which have been associated with adverse biological effects, one should of course not fully ignore the possibility that CLA may have substantial anti-obesity properties and/or that it has (other) beneficial effects that may override the subtle adverse effects that have been identified. Many (non-natural) drugs, including e.g. statins do have different degrees of adverse effects, but their beneficial effects clearly overrides their adverse effects – and so could CLA.

In conclusion, the current evidence on the effects of CLA on obesity and associated risk factors are highly controversial. Therefore, before any recommendations should be put forward stating that CLA-supplementation can be taken without any safety concerns, further studies are needed, preferably including a large sample size and including accurate measures of changes in body composition and various risk markers associated with the development of MS, T2D and CVD.

3. List of abbreviations

ADA	American Diabetes Association
ADIGEN	ADIposity and GENetics
AFABP	Adipocyte lipid-Binding Protein
Apo(b)	Apoliprotein B
BMI	Body Mass Index
BNP	Brain Natriuretic Peptide
CARLA	Co-Activator-dependent Receptor Ligand Assay
CD/CV	Common Disease/Common Variant
CLA	Conjugated Linoleic Acid
CRP	C-Reactive Peptide
CT	Computed Tomography
CVD	Cardiovascular Disease
DGAT-1	Diacylglycerol O-acyltransferase 1 protein
DHA	DocosaHexenoic Acid
dHPLC	Denaturing High-Performance Liquid Chromatography
DXA	Dual X-ray Absorptiometry
EGIR	European Group for the study of Insulin Resistance
EPA	EicosaPentanenoic Acid
FDA	Food and Drug Administration
FFA	Free Fatty Acids
HbA1c	Hemoglobin A1c
HDL	High Density Lipoprotein cholesterol
HIV	Human Immunodeficiency Virus
HMGI-C	High Mobility Group protein
HOMA	Homeostasis model
HOMA-IR	Homeostasis Insulin Resistance model
hsCRP	hypersensitive C-Reactive Peptide
HSL	Hormone Sensitive Lipase
ICAM	InterCellular Adhesion Molecule
IL	InterLeukine
IMCL	Intra-MyoCellular Lipid
IMT	Intima-Media Thickness
KO	Knock Out
Lipin-1	Fatty liver dystrophy protein
LDL	Low Density Lipoprotein cholesterol
LP(a)	LipoProtein a
LPL	LipoProtein Lipase
MCP-1	Monocyte chemotactic protein 1
MC4R	MelanoCortin-4 Receptor
MMP	Matrix MetalloProteinase
MR	Magnetic Resonance
MS	Metabolic Syndrome
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic SteatoHepatitis
NCEP/ATPIII	National Cholesterol Education Program/Adult Treatment Panel III
NHANES	National Health and Nutrition Examination Survey
NHR	Nuclear Hormone Receptor
PAI-1	Plasminogen activator inhibitor type-1
PEPCK	PhosphoEnolPyruvate CarboxyKinase protein
POMC	Pro-OpioMelanoCortin
PPARγ	Peroxisome Proliferator-Activated Receptor gamma

Pref-1	Preadipocyte factor-1
P:S ratio	Ratio of dietary intake of polyunsaturated fat to saturated fat
QTL	Quantitative Trait Loci
RXR	Retinoic acid Receptors
sCD40L	soluble CD40 Ligand
SFA	Saturated Fatty Acids
SGBS	Simpson-Golabi-Behmel Syndrome
SNP	Single Nucleotide Polymorphism
TFA	Trans Fatty Acids
TG	Triglycerides
TNFα	Tumor Necrosis Factor alfa
TTA	Tetradecyl Thioacetic acid
TZD	Thiazolidinedione
T2D	Type-2 Diabetes
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein
WAT	White Adipose Tissue
WHO	World Health Organisation

4. References

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Due to restrictions from the publishers of the journals in which paper 1, 3 and 4 have been published, these papers are not present in this PDF. The papers can be found in:

Paper 1:
Larsen, T. M., Larsen, L. H., Torekov, S. K., Ek, J., Black, E., Toubro, S. et al. (2005). Novel Variants in the Putative Peroxisome Proliferator-activated Receptor γ Promoter and Relationships with Obesity in Men. *Obesity, 13,* 953-958.
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Paper 3:
Larsen, T. M., Toubro, S., & Astrup, A. (0 AD). PPARgamma agonists in the treatment of type II diabetes: is increased fatness commensurate with long-term efficacy? *Int J Obes Relat Metab Disord, 27,* 147-161.
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Paper 4:
Larsen, T. M., Toubro, S., & Astrup, A. (2003). Efficacy and safety of dietary supplements containing CLA for the treatment of obesity: evidence from animal and human studies. *Journal of Lipid Research, 44,* 2234-2241.
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One year conjugated linoleic acid supplementation does not prevent weight or body fat regain.

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Running title: CLA supplementation for the treatment of obesity

ABSTRACT

Background

Conjugated linoleic acid (CLA) is marketed as a safe, simple and effective dietary supplement to promote body fat and body weight loss. However, most of the previous studies have been of short duration and inconclusive, and some recent studies have questioned the safety of long term supplementation with CLA.

Objective

To assess the effect of one year supplementation with CLA (3.4g/day) on body weight and body fat regain in moderately obese people.

Design

122 obese (BMI>28 kg/m^2) healthy subjects underwent an 8-week dietary run-in with energy restriction (3300-4200 kJ/day). 101 subjects who lost >8% of initial body weight, were subsequently randomised to a 1 year double blinded CLA (3.4g/day, n=51) or placebo (olive oil, n=50) supplementation regime in combination with a modest hypocaloric diet of -1250 kJ/day. Treatment effects on body composition using Dual-energy X-ray Absorptiometry (DXA) and safety (blood samples, adverse events) were assessed.

Results

After 1 year no significant difference was observed between treatments. The CLA group (n=40) regained 3.1 ± 5.3 kg body weight and 2.1 ± 5.0 kg fat mass compared with 3.2 ± 5.2 kg and 2.7 ± 4.9 kg, for body weight and fat mass respectively, in the placebo group (n=43). There were no differences in reported adverse effects or indices of insulin resistance, but a possible incremental effect on leucocytes was observed.

Conclusion

One year 3.4 g daily CLA supplementation does not prevent weight or fat mass regain in a healthy obese population.

Key words: Conjugated linoleic acid, dietary supplement, obesity, body fat, safety

Introduction

The long-term effects of conventional weight-management programmes are unsatisfactory, and alternative therapies, including dietary supplements, are repeatedly called for by the obese and the society. Although the use of dietary supplements is widespread, their documentation on efficacy and safety is not convincing (1). Conjugated linoleic acid (CLA) is a mixture of linoleic acid isomers with conjugated double bonds which has been studied intensively(2). CLA is sold commercially as dietary supplements for weight and/or fat loss. The products often have a 40/40 % content of cis9, trans11 (c9,t11), trans10,cis12 (t10,c12) fatty acids and the remaining 20% are usually other fatty acids; approx. 1-4 % other types of conjugated fatty acids and 15-19 % other non-conjugated fatty acids (3). The CLA isomer c9,t11 is a natural constituent in the human diet and the average daily intake of the c9,t11 isomer in western societies is between 150-200 mg/day(4), whereas the intake of the t10,c12 isomer is negligible.

In humans, a dose-response study with CLA (a mixture of c9,t11 and t10,c12) over 3 months reported a decrease in body fat, assessed by DXA-scanning, without additional effect at doses greater than 3.4 g/day (5). The only published long-term (>6 months) placebo controlled human study provided subjects with CLA (a mixture of c9,t11 and t10,c12 as either free fatty acids or triacylglycerol) for 12 months and induced significant losses of 2.0 kg body weight (CLA triacylglycerol group only) and 2.2 kg body fat mass (both CLA supplementation regimens) compared to placebo (6). An open label 1-year extension of that study showed that the placebo group lost body fat mass when given CLA, but also that 2-year treatment did not add further loss of fat mass compared to 1 year treatment (7).

To our knowledge, only two studies have investigated the effect of CLA during weight gain in humans, and both found no effect on body weight or body fat regain after 3 or ~6 months treatment with CLA (a mixture of c9,t11 and t10,c12) after an initial loss of body weight(8;9). However, the 3 months study found that CLA did increase the amount of lean body mass when

compared to placebo(8).

In the present study, we investigated whether one year supplementation with CLA (3.4g/day of a mixture of c9,t11 and c10, t12 as triacylglycerols) could decrease body weight and body fat mass regain in moderately obese people after a Low Calorie Diet (LCD) induced weight loss. In addition we assessed treatment safety (monitoring adverse events, vital signs, ECG, and blood parameters including indices of insulin resistance) and the influence of CLA on hormones that may influence growth and/or body fat metabolism such as insulin-like growth factor-1 (IGF-1), growth hormone (GH), thyroid-stimulating hormone (TSH) and testosterone.

Subjects and methods

The study was initiated in January 2002, the first subject was included in February 2002, and the clinical part of the study was terminated in June 2003. Subjects were healthy participants of both genders between 18 and 65 years of age with BMI between 28 and 35 kg/m^2. The subjects were recruited by 2 research centers (Department of Human Nutrition, RVA University, Copenhagen, Denmark & Department of Clinical Nutrition Hvidovre Hospital, Copenhagen, Denmark). All subjects gave written informed consent before inclusion in the study. Subjects were not included if they did not have a stable weight (± 3 kg in the last two months), were receiving drug therapy, consuming a special diet, or taking dietary supplements for weight loss. In addition, pregnant or lactating women were excluded. Subjects with renal, liver, pancreatic, or chronic inflammatory or infectious diseases, cardiac disease or malignant tumors were excluded. Diet treated diabetics as well as subjects with treated simple hypertension were allowed inclusion. Subjects who had active thyroid disease or who were receiving thyroid hormone treatment, and subjects taking adrenergic agonists, or with known or suspected drug or alcohol problems or with any clinical condition rendering them unfit to participate were excluded.

The study was a randomised double-blinded, placebo controlled, parallel-group study with two

treatment arms. Initially, all subjects followed a LCD (Nutrilett®, Collett Pharma, Lysaker, Norway), with an energy content of 3300-4200 kJ/day for 8 weeks. All subjects attended 6 group meetings where experienced dieticians guided them through the weight loss regimen. Subjects that lost 8% or more of their initial weight during the LCD were randomised to CLA or placebo. The two treatment groups received either 6x750mg capsules of CLA (TONALIN®, Natural ASA, Hovdebygda, Norway) or 6 capsules of placebo (4.5 g olive oil) per day. The dose was selected based on previous studies(5;10). The CLA content of the capsules was approximately 80% of total lipid (~3.4g CLA/day); 39% c9,t11 CLA & 41% t10,c12 CLA as triacylglycerols and 20% others, as analysed by the manufacturer. The soft gel capsules were opaque and identical in taste and in appearance, and the energy content in both types of capsules was matched. The randomization sequence was generated by Scandinavian Clinical Research (Contract Research Organization) using a simple block randomization procedure without any stratifications. The allocation sequence was provided to each centers study personnel via an internet based interface. Both centers followed the study's randomization procedure and did not break the code at any time during the study. The randomization list was kept confidential and was opened only after closure of the database. Assuming a difference of 1.7 kg of fat mass between treatment groups based on prior studies of 3.4 g CLA/day vs. placebo(5) and an estimated SD of 2.2 kg (based on prior experience of within-group detectable differences using DXA-methodology) an estimated number of 37 subjects pr. group were required (using 90% statistical test power and 5 % significance level). To account for a high dropout rate, the required number of subjects needed for initial inclusion was estimated to be N=60 per group. After the 8-week LCD-period (Week 0), the 101 subjects who achieved the weight loss goal were randomised. For a total of 52 weeks, subjects were provided with CLA or placebo in combination with a modest hypocaloric diet of approx. -1250 kJ/day (1 kcal = 4.18 kJ). The dietary instruction was given via 14 individual consultations (~1 per month) from dieticians throughout the treatment period and was based on the isocaloric interchangeable

and educational diet program "Eat for life"(11). The energy requirement during the 52 week hypocaloric period was estimated using appropriate equations, according to body weight, gender and age(12).

Characteristics (including smoking and drinking habits) and demographic data were recorded when subjects entered the study. Body weight, adverse events and concomitant medication were recorded at each visit with the dietician, i.e. a total of 14 times during the 52 weeks treatment. Physical measurements, blood samples, urine samples, measurements of blood pressure, waist, hip, pulse, and ECG were taken and DXA scanning was performed 4 times in total, i.e. before the LCD-period (Week -8), after the LCD-period (Week 0) and after ~26 and 52 weeks of treatment. The dietary supplements were provided to (and returned from) the study participants at every bimonthly visit. Fasting blood samples (>10 hours fasting) were obtained at between 07.00-13.00 hours, and the time point of sampling was repeated if possible. The samples were all (except insulin) analysed in an accredited laboratory (Capio Diagnostik, Copenhagen, Denmark). Insulin was analysed using a commercially available kit (DPC, IMMULITE 1000 Insulin).

Blood was analysed for the following: Haemoglobin, erythrocytes, leukocytes (white blood cells), platelets, alanine aminotransferase, aspartate aminotransferase, gamma glutamyltransferase, creatinine, IGF-1, GH, TSH, glucose, insulin and total testosterone. An index of insulin resistance (HOMA-R), was derived from fasting values of glucose and insulin according to the formula: Glucose*Insulin/22.5. Urine was obtained for analysis of blood, glucose and protein content and for pregnancy testing. All blood samples were taken, and DXA-scanning and clinical assessments (except body weight) were performed at the same center, i.e. the Royal Veterinary and Agricultural University, Copenhagen. Blood samples were obtained at all time points for a total of 75 participants. Compliance was measured every 2 months by comparison of the number of unused capsules with the number of capsules that should have been used. A subject was considered compliant when he or she took $\geq$ 75% of the capsules provided.

DXA-scanning (Lunar Radiation Corp, Madison, WI) was used to determine body composition with LUNAR PRODIGY software (version 5; Lunar Radiation). All DXA-scans were performed by the same experienced technical assistant in the morning within +/- 1 hour for each subject. The results of the DXA-scan were divided into body fat mass (FM) and body fat free mass (FFM). Fat free mass was calculated as lean tissue mass + bone mineral content. Fat mass percentage is calculated as the ratio fat mass (DXA)/(fat mass (DXA) + fat free mass (DXA))*100. Waist and hip circumferences were measured immediately before DXA-scanning. Body weight was assessed at each visit on regularly calibrated electronic weight scales (SCALE). In addition body weight was also assessed as the sum of fat mass and fat free mass as assessed by DXA-scanning at 4 time points during the study.

Diet records were completed 3 times: before entering the study (week -8) and after ~24 and 52 weeks of treatment. Each participant recorded their diet for 3 consecutive days prior to the visit at the medical center according to a previously evaluated method (Astrup, 2002). The method provides information on quantity and type of all food consumed during the 3-day registration period. Each participant was given detailed instructions on how to fill out the questionnaire, and all returned questionnaires were monitored by the clinical dietician. Food intake was converted into energy intake.

Ethics

The study was approved by the regional Ethics Committee for the districts of Copenhagen & Frederiksberg, Denmark (journal no. KF 01-247/01). The study was performed according to the Declaration of Helsinki (Edinburgh Amendment 2000) and the current International Conference on Harmonization (ICH) guidelines.

Statistics

All analyses were performed using the SPSS® 12.0 software. Statistical tests were performed using 5% as the nominal level of significance and interval estimates were constructed using 95% as the level of confidence. We defined a modified Intention-To-Treat (ITT) population (Modified ITT = 77 patients who completed the 26 week treatment + 6 subjects who were withdrawn or lost during the last 26 week treatment, i.e. a total of 83 subjects) which were used for the analysis of changes from baseline to 1 year. Use of last-observation-carried-forward (LOCF) was used for this modified ITT population who completed the 26 weeks treatment, but did not complete the 1 year period, for the analyses of changes in fat mass, fat free mass, fat percentage and body weight. Also LOCF was applied on missing values in the 8-week LCD period for body weight. No substitution of missing data was performed on other variables. No stratification was used. Fisher's exact test was used for testing differences between treatment groups regarding gender distribution and drop out rate. Analysis of Covariance (ANCOVA) was used to compare the changes in fat mass, fat free mass, fat percentage and body weight in the CLA and placebo groups using the week 0 value and the body weight change (weight at week 0 minus weight before LCD) as covariate. Also treatment center and gender was inserted as covariates. We also performed repeated analyses of changes in fat mass, fat free mass, fat percentage and body weight. The values for each variable at the time points week 0, 25 and 52 was compared between treatment groups, using change in body weight at week 0 minus weight before LCD, center and gender as covariates. The changes from week 0 to week 52 within treatment groups were tested with the paired T-test. Between group analyses of changes in waist, hip, energy intake and all blood parameters were analyzed using an unpaired t-test.

Results:

135 subjects were screened for potential inclusion in the study and 122 subjects were enrolled in

the 8 week LCD-period. A total of 101 subjects were randomized to one of the two treatment groups after having completed the 8 week LCD and lost at least 8% of their initial body weight. The 83 subjects who completed the 26 weeks of treatment were included in the 1-year (modified) intention to treat analyses (ITT), i.e., 40 subjects in the CLA group and 43 subjects in the placebo group. 77 subjects completed the whole treatment period. **[INSERT FIGURE 1]** As assessed by the counting of returned capsules, the compliance was high: 95.7 $\pm$ 8.7 % and 96.7 $\pm$ 8.8 % for the CLA and placebo group respectively (NS). The drop out rate (CLA: 27.5% vs. placebo: 26.0 %) did not differ significantly between the treatment groups after 12 months.

Baseline characteristics

The subjects were all of Caucasian origin and the study groups were well matched with respect to gender, ethnic origin, smoking habits (n=83), habitual alcohol intake and body height (data not shown). There was no difference between groups at the start of the study with respect to vital signs (blood pressure and heart rate), medical conditions or concomitant medications. Significant differences between the groups ($p<0.05$) were observed for body weight, BMI, body fat mass and lean body mass at week –8 (before LCD) and week 0 (baseline). Subjects in the placebo group had higher body weight and BMI than subjects in the CLA group. Consequently, body fat mass and fat free mass were also higher in the placebo group than in the CLA group. However, the percentages of body fat mass were not different between the groups at week -8 and at week 0 (baseline). Hip measurements were slightly higher in the placebo group at baseline, whereas waist and waist/hip measurements were not different between treatment groups **[TABLE 1].**

Changes in body weight, body composition & dietary registrations

During the LCD period, both groups of subjects lost about 10 kg body weight of which ~70% was fat mass, group difference was not significant **[TABLE 1]**. According to the food records, energy

intake decreased from baseline to week 52 in both groups despite an average weight gain during the same period. No significant differences between groups were found; during the one-year supplementation period, both groups regained approximately 3 kg body weight when measured by DXA-scan (data not shown) and approximately 4 kg when assessed on a weight scale **[FIGURE 2]**. Similarly, both groups gained equal amounts of fat mass; 2.1 + 5.0 kg vs. 2.7 + 4.9 kg and fat free mass 0.9 + 1.7 kg vs. 0.5 + 1.8 kg in the CLA and Placebo groups, respectively. Repeated analyses provided essentially the same results ($p = 0.65$ for changes in body fat mass). It should be noted that the LOTF analysis is rather conservative, making it less likely to detect a treatment effect (the drop outs are more likely to be the individuals who could not maintain the weight loss and therefore the reported mean weight gain data are probably too low). Changes in hip and waist measurements did not differ between groups at any time point.

Adverse events (AEs)

A total of 563 adverse events were reported, but with no significant difference between the two groups. Among these AEs, 35 were considered related to the treatment and 528 were not. 94.1% and 98.0% of subjects from CLA and placebo group respectively experienced AEs during the study (not significant between groups). 3 subjects from the CLA group and 2 from the placebo group withdrew from the study because of pregnancy of because of adverse events (soft stools, depression, air in stomach or stomach pain). 5 serious AEs were registered, 4 of them in the placebo group. None of them were considered related to the treatment.

Laboratory analyses

Several of the safety blood parameters changed during the LCD induced weight loss, but these parameters recovered during the subsequent weight regain period (week 0 until week 52), and to a similar extent in both groups (data not shown). Only the change in leukocyte concentration from

week 0 to week 52 was significantly different between groups with a higher increase in the CLA group (0.81 ± 1.21 10^9/L) than placebo (0.19 ± 1.14 10^9/L, p=0.033). This increase appeared only after more than 25 weeks of supplementation. Urine analyses, ECG registrations, systolic and diastolic blood pressure and heart rate did not reveal any other abnormal observations in any of the study groups (data not shown). CLA did not affect fasting values of plasma glucose and insulin, and insulin resistance was also not affected, as assessed by the HOMA-R index **[TABLE 2]**. In addition to the safety parameters, we assessed a panel of hormones (Testosterone, GH, IGF1 and TSH), but CLA treatment did not affect any of these measures **[TABLE 2**].

Discussion

Numerous animal studies have shown that CLA causes repartitioning of the body composition i.e. decreased fat mass and increased lean body mass(13). In addition, CLA has been reported to be an effective inhibitor of atherogenesis in rabbits(14) and of insulin resistance in skeletal muscle in rats(15). Hence, CLA has been suggested to be useful in treating diabetes by controlling body fat and weight gain(16), but other recent studies in humans have indicated that CLA may actually have negative effects on insulin sensitivity(17). Though the animal studies have been very optimistic, and some human studies have also shown positive effects on body fat, most human studies (duration <6 months) have shown only marginal effect on body weight and body composition (18). The apparent discrepancies between animal and short-term human studies have been ascribed to a) lower doses pr kg body weight, b) shorter treatment duration in human studies, c) the view that CLA may only be effective during fat accumulation and d) the quality and reliability of methods of measurements.

Our main objective was to investigate whether one year of CLA supplementation in obese adults would safely prevent regain of body fat mass and body weight after a major initial weight loss as compared to placebo. In this respect, we found no effect on either body weight, body fat mass or

fat free mass. The absence of a significant effect on fat mass and body weight corroborates the results of other studies recently reported. In a placebo controlled study, obese subjects were given 6g CLA/d for 28 weeks, the first 12 weeks being in conjunction with a LCD(9). CLA neither affected body weight nor body fat content(9). Also, Kamphuis et al. performed a study with a CLA preparation similar to that used in our study and found that neither of 2 CLA doses (1.8g/day and 3.6g/day) were able to significantly lower fat mass and body weight when supplemented for 13 weeks, after an initial ~6 kg weight loss(8). However, CLA did significantly decrease the regain of fat free mass by ~2.3 %. Our findings stand in contrast to the findings from Gaullier et al. which performed a 1 year study in a large sample of moderately obese subjects given 3.4 g CLA/day(6). Here CLA supplementation induced a significant weight and body fat loss of 2.0 and 2.2 kg, respectively. The discrepancy between the studies is not easily explainable, but could be due to the different designs, i.e. our study was carried out in more obese persons and it was performed during weight gain, whereas the study by Gaullier et al. was not. Another explanation may be the absence of diet restrictions during the study by Gaullier et al. A third possible explanation is the significant baseline differences between the groups in the present study. However, by using the ANCOVA analysis this difference is not likely to have influenced the study to any major extent, but we also note that, given the finding of a numerical difference of approx. 0.6 kg fat mass between groups and a SD of ~5 kg, the present study may have been inadequately powered.

The secondary endpoint in the current study was the safety issue. Generally, CLA supplements have been considered to be safe when studied in animals(19). However, some studies in mice have shown that particularly the t10,c12 isomer may induce lipoatrophy, hyperinsulinaemia and fatty liver (20) and that this may occur at dosing levels that are comparable to the doses used in human studies(21). However, due to species differences, it should be emphasized that one should always be cautious when comparing human and animal studies. In this respect, there are also human

studies that have questioned the safety of commercially available supplements of CLA, by reporting that the t10,c12 isomer (and perhaps the cis9,trans11 isomer) produce insulin resistance, even when taken for only 4 months(22;23). In contrast, a 1 year study showed that CLA does not seem to impair glucose metabolism or liver function, though it may increase HDL, LDL and apolipoprotein B in some circumstances(6). In the present study CLA did not affect fasting values of plasma glucose and insulin, and we did not find any effect on HOMA-R. This confirms previous studies by Riserus et al. where only supplements with purified trans-10, cis-12 (22) or cis-9, trans-11 isomers (23) increased insulin resistance among subjects with the metabolic syndrome, whereas a 50/50 mixture like the one used in the present study apparently did not(22). In the present study, 21% of the reported AE´s were severe, with 7% and 5% related to CLA and placebo, respectively, NS. The majority of the AE´s were related to the gastrointestinal tract. The number of drop-outs was low for a one-year study with obese subjects (23.7%, without difference between groups). The compliance was very high (96%), indicating that 6 capsules/day for 1 year was no source of discomfort. Some minor changes in the laboratory safety data were observed in both groups in one direction during the LCD period, and in the opposite direction during the one-year supplementation, but without any difference between CLA and placebo. Also, the urine tests and the ECG records were normal until the end of the study. Only leukocyte concentrations was increased by 1 year CLA treatment. Similar observations have been reported in previous long-term studies(6;7;9). Although the actual increase in leucocytes is generally small, this increase may be of some concern as previous studies have indicated that leukocytes are an important indicator of inflammation and has also been identified as an predictor of CHD mortality(24). As the values are within reference values [3.0-10.0 10^9/L], and as CLA in most cases are used for a limited time period, the clinical relevance of this finding is still unclear. Also, a recent 12 week study in humans actually suggested that CLA may have beneficial effects on immune function(25). Further studies should be done to clarify this issue. We analysed whether CLA

might affect energy metabolism via effects on the hormones testosterone, GH, IGF1 or TSH. We found no changes, which seems to be in accordance with the apparent lack of effect on body weight and body composition.

Conclusions

Unfortunately we did not obtain a perfect group match for body weight at randomization. Despite the reservations that this issue may imply, we conclude that 1 year of supplementation with a mixture of 3.4 g/day CLA isomers has no clinically important effect on body weight and body fat regain after an 8 week LCD induced weight loss.

Acknowledgements

We thank the staff of the Department of Human Nutrition, RVA University, Copenhagen, Denmark and Department of Clinical Nutrition Hvidovre Hospital, Copenhagen, Denmark. The author's contributions to this publication are as follows: collection of data, data analysis, interpretation and co-drafting (TML). Study initiation, design, collection of data, data interpretation and co-drafting (ST). Initiation, design, data analysis, interpretation and co-drafting (OG). Initiation, design, data interpretation and co-drafting (AA). The sponsor of the study had no role in data collection, data analysis, data interpretation, the writing of the report, or the decision to submit the report for publication. None of the authors had any personal or financial conflicts of interest to report.

Table 1. Changes in body weight, body composition, waist, hip and energy intake.

		Before LCD[1,2]	Week 0 (Baseline)	Week 25	Week 52[3]	Δ 52-0	Δ 52–0 p-value within group[4]	Δ 52–0 p-value between groups[5]
Weight (kg)	CLA	94.5 ± 11.7	82.6 ± 9.5[6]	84.6 ± 11.3	86.7 ± 13.2	4.0 ± 5.6	p < 0.001	0.51
	Placebo	100.6 ± 13.2	88.5 ± 12.0	90.7 ± 13.0	92.5 ± 13.0	4.0 ± 5.0	p < 0.001	
FM (kg)	CLA	34.6 ± 6.9	26.9 ± 7.2[7]	26.6 ± 7.5	29.0 ± 8.5	+ 2.13 ± 4.99	p = 0.01	0.56
	Placebo	37.4 ± 7.4	29.9 ± 8.1	29.7 ± 9.5	32.6 ± 9.6	+ 2.73 ± 4.92	p < 0.001	
FFM (kg)	CLA	59.3 ± 11.5	56.4 ± 11.1	57.9 ± 11.5	57.4 ± 11.7	+ 0.94 ± 1.74	p < 0.001	0.33
	Placebo	62.3 ± 12.8	59.7 ± 12.2	60.7 ± 12.3	60.2 ± 12.3	+ 0.51 ± 1.77	p = 0.066	
FAT % [DXA][8]	CLA	37.1 ± 7.3	32.5 ± 9.0	31.6 ± 8.5	33.7 ± 8.7	1.1 ± 4.0	p = 0.086	0.47
	Placebo	37.9 ± 7.5	33.7 ± 8.7	33.0 ± 9.5	35.2 ± 9.2	1.4 ± 3.9	p = 0.008	
Waist (cm)	CLA	101.9 ± 8.8	93.3 ± 7.8	95.0 ± 7.1	97.5 ± 8.95	4.2 ± 5.9	p < 0.001	0.88
	Placebo	104.9 ± 9.8	95.8 ± 9.6	98.6 ± 10.4	99.7 ± 10.8	4.0 ± 6.7	p < 0.001	
Hip (cm)	CLA	108.3 ± 7.8	101.9 ± 6.5[9]	104.2 ± 6.3	106.7 ± 7.3	4.9 ± 6.3	p < 0.001	0.37
	Placebo	109.6 ± 7.5	104.7 ± 6.5	108.3 ± 8.1	110.7 ± 7.9	6.1 ± 5.9	p < 0.001	
Energy intake (KJ/day)	CLA	10102 ± 3238	-	7167 ± 1999[10]	7807 ± 2054	-2295 ± 2690	p < 0.001	0.71
	Placebo	9889 ± 2378	-	7567 ± 2461[10]	7328 ± 1622	- 2561 ± 1981	p < 0.001	

[1]The number of subjects were CLA=40 and Placebo=43.

[2]The gender distribution was male/female (CLA=17/23, Placebo=19/24) and the age was (mean+SD, CLA=43.4 ± 8.4, Placebo=41.7 ± 8.2 years), with no significant difference between groups.

[3]For body weight, fat mass, fat free mass and FAT%, Last-Observation-Carried-Forward analysis is applied for subjects who completed the 26 weeks treatment, but did not complete the 1 year period.

[4]Paired t-test

[5]For analyses of changes in weight, FM, FFM and FAT%, covariance analysis (ANCOVA) was performed using the week 0 value and the body weight change (week 0 minus week -8), treatment center and gender as covariates. Other changes was analysed using unpaired t-test.

[6]Values are mean +/- SD

[7]The change in body weight (kg or %) and the change in FM, FFM and FAT% during the 8 week LCD was not different between the groups.

[8]Fat mass percentages are calculated as the ratio FM(dxa) / (FM(dxa)+FFM(dxa))*100.

[9]Significantly different at baseline ($p<0.05$)

[10]Assessed at week 24, the number of subjects reporting energy intake at all time points was CLA=28, Placebo=20.

346 **Table 2. Blood parameters before LCD, at week 0, week 25 and week 52. n(CLA)=36, n(placebo)=39**.

Blood parameter **Absolute values**		**Before LCD**	**Week 0 (Baseline)**	**Week 25**	**Week 52**	**Δ 52-0**	**Δ 52–0 p value between groups[1]**
Glucose (mmol/l)	CLA	5.03 ± 0.38[2]	4.79 ± 0.44	4.94 ± 0.36	4.94 ± 0.47	0.16 ± 0.73	0.198
	Placebo	5.03 ± 0.50	4.92 ± 0.51	4.85 ± 0.48	4.87 ± 0.47	-0.4 ± 0.61	
Insulin (pmol/l)	CLA	60.2 + 19.1	40.6 + 16.0	44.1 ± 20.3	53.5 ± 21.8	12.9 ± 18.9	0.221
	Placebo	58.4 + 23.0	44.2 + 23.4	42.9 ± 20.8	50.9 ± 24.4	6.66 ± 24.2	
Growth horm. (µg/l)	CLA	0.60 ± 0.76	1.20 ± 2.40	1.15 ± 2.34	0.50 ± 0.69	-0.70 ± 2.12	0.982
	Placebo	0.50 ± 0.72	1.59 ± 3.18	0.98 ± 1.61	0.87 ± 1.54	-0.72 ± 3.49	
IGF-1 (nmol/l)	CLA	20.2 ± 5.8	20.0 ± 6.9	24.1 ± 7.4	23.9 ± 5.4	3.71 ± 6.84	0.465
	Placebo	22.5 ± 6.9	19.6 ± 6.6	24.5 ± 6.5	24.5 ± 6.9	4.75 ± 4.86	
Testosterone (nmol/l)	CLA	6.91 ± 6.50	8.60 ± 7.91	9.20 ± 8.19	6.52 ± 6.74	-2.09 ± 2.77	0.151
	Placebo	6.78 ± 6.16	8.25 ± 7.05	9.65 ± 8.51	7.14 ± 7.53	-1.10 ± 3.05	
TSH (mIU/l)	CLA	1.33 ± 0.49	1.20 ± 0.58	1.41 ± 0.59	1.07 ± 0.36	-0.13 ± 0.44	0.867
	Placebo	1.33 ± 0.63	1.24 ± 0.64	1.44 ± 0.90	1.12 ± 0.63	-0.11 ± 0.45	
HOMA-R[3]	CLA	13.7 ± 4.7	8.8 ± 4.0	9.9 ± 5.1	11.8 ± 4.7	3.01 ± 4.63	0.119
	Placebo	13.2 ± 5.7	9.9 ± 5.9	9.3 ± 4.6	11.0 ± 5.2	1.10 ± 5.72	
Leukocytes (10^9/L)	CLA	6.21 1.43	5.56 1.69	6.03 1.39	6.36 1.66	0.81 ± 1.21	0.026
	Placebo	5.85 1.53	5.32 1.53	5.89 1.54	5.51 1.30	0.19 ± 1.14	

347 [1] Unpaired t-test.

348 [2] Values are mean +/- SD

349 [3] HOMA-R=glucose*insulin/22.5

Figure legends and footnotes

Figure 1: Study participant & randomization scheme

Footnotes: LCD indicates the initial 8 week low calorie diet period
ITT indicates the study population for which Intention-To-Treat analyses are performed for changes in body weight, body fat, fat free mass and body fat percentage from baseline to 1 year treatment (n=83 in total). The dropout rate was not significantly different between groups (χ^2 test).
AE = Withdrawn due to Adverse Event

Figure 2: Body weight during the study.

Footnotes:
Number of subjects are CLA=40, Placebo=43.
Where relevant, Last-Observation-Carried-Forward has been used to insert body weight for week 26 (or later) to be inserted at week 52.
There was no significant difference between groups in body weight change throughout the study (both $p > 0.5$ using ANCOVA or repeated measures analysis).

Error bars indicate SEM

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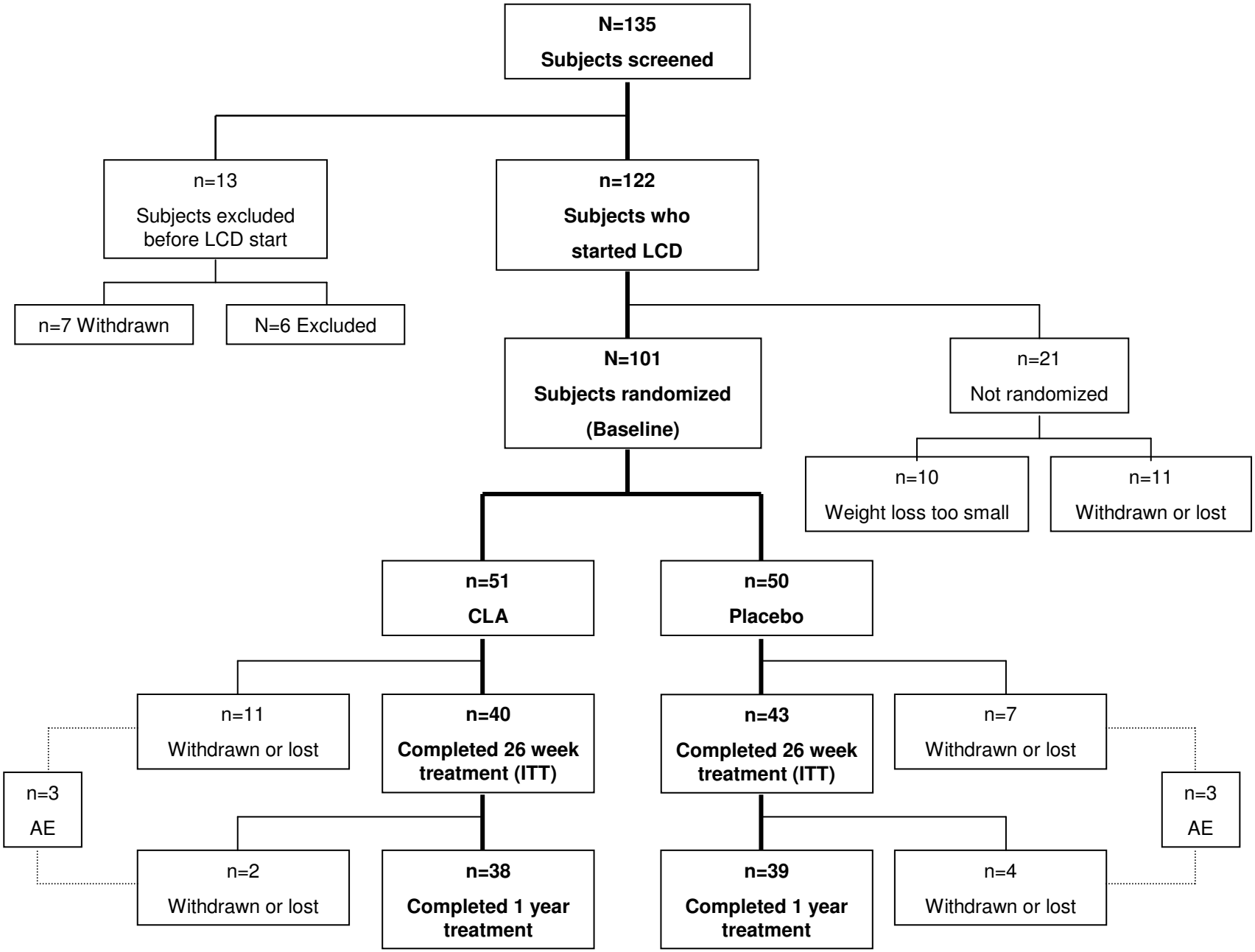
N=135
Subjects screened
n=13
Subjects excluded before LCD start
n=122
Subjects who started LCD
n=7 Withdrawn
N=6 Excluded
N=101
Subjects randomized (Baseline)
n=21
Not randomized
n=10
Weight loss too small
n=11
Withdrawn or lost
n=51
CLA
n=50
Placebo
n=11
Withdrawn or lost
n=40
Completed 26 week treatment (ITT)
n=43
Completed 26 week treatment (ITT)
n=7
Withdrawn or lost
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n=38
Completed 1 year treatment
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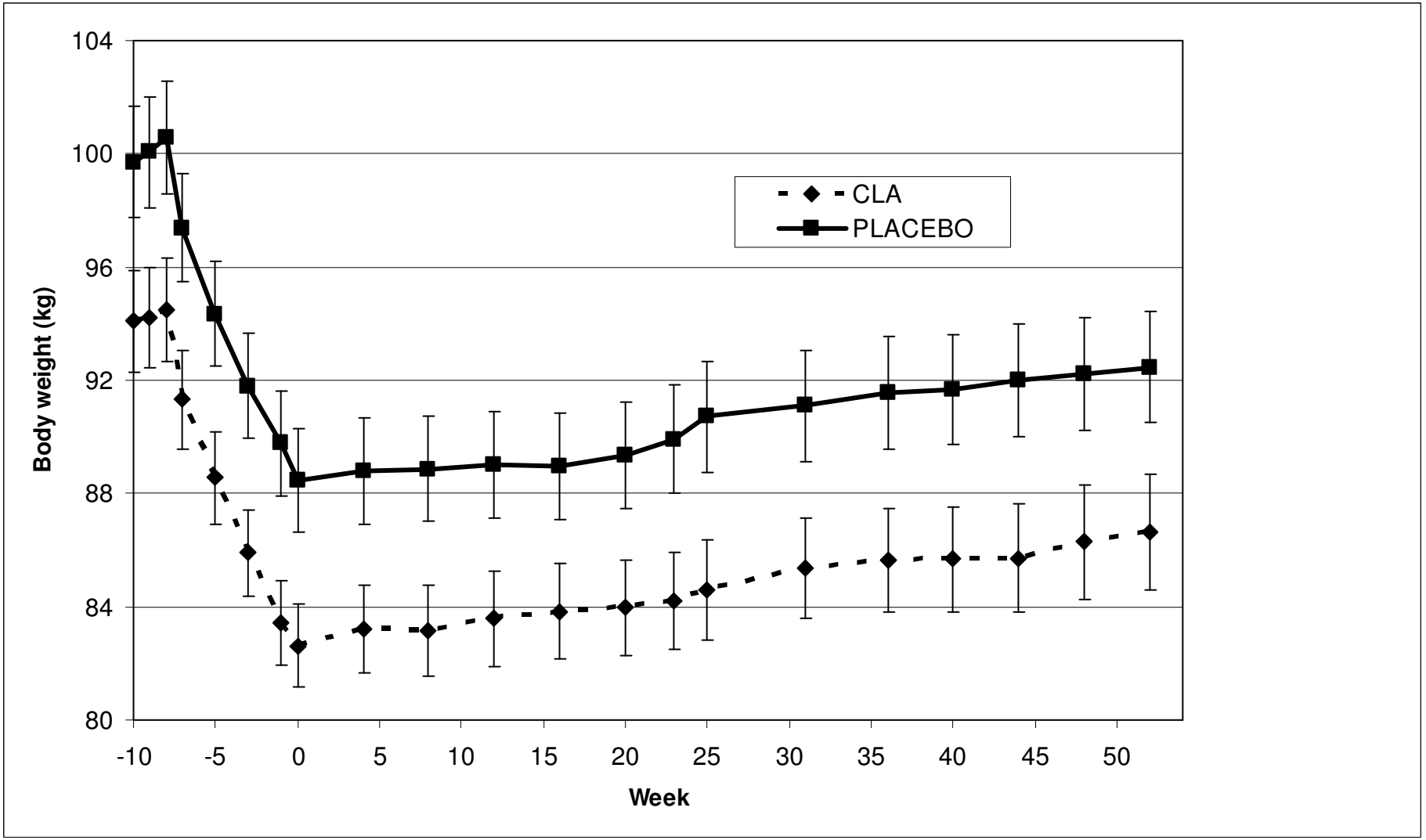
Withdrawn or lost


104
100
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Body weight (kg)
CLA
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Week

Nov 3, 2004

To Associate Editor
Lee M. Kaplan, MD, PhD,
Obesity Research

Re: Revised version of MS# 04-113
Identification of novel variants in the putative PPARγ2 promoter and relationships with obesity in Danish men

Dear Dr. Kaplan,
We are most grateful for the constructive comments from You and the reviewers, and we have done our best to comply with them within the space limits of this type of reports.

Editor corrections/manuscript formatting

The present manuscript has been corrected according to the "Brief Genetic Analysis" format, i.e.

A) No headings for Introduction, Results, or Discussion.
B) Methods section follows main text.
C) Accordingly, we have changed the order of the tables 1, 2 and 3.

Ekstra notes

The reference number 1 has been changed:

From: *Allison DB, Kaprio J, Korkeila M, Koskenvuo M, Neale MC, Hayakawa K*. The heritability of body mass index among an international sample of monozygotic twins reared apart. Int.J.Obes.Relat Metab Disord. 1996;20:501-6)

To: *Sorensen TIA*. The genetics of obesity. Metabolism 1995;44:4-6.)

Point to point reply to reviewers.

Reviewer 1

We are most grateful for the helpful and constructive criticism offered by the reviewer.

Q1 (p7, para3): This appears to be a retrospective cohort identified from military records. To do the genotyping a blood sample must have been obtained recently. State briefly what the participation rate was of the originally chosen subjects in the follow-up and blood sampling. A statement about informed consent or ethics committee approval should be made.

Reply: The study is a retrospective study, and the blood samples have been obtained recently (year 1998-2000). We have now made it clear how the current study group relates to the original epidemiological study population of draftees, page 10 line164. In the paper, we now also provide the informed consent statement and the ethics committee approval as required by the reviewer, page 10 line 177. Also the participation rate is provided, page10 line 174

Q2 (p8, para3):
Exact tests are appropriately used for categorical variables. It appears for the data on genotype vs BMI (p.10) that ANCOVA was used on highly skewed data from the very small numbers (e.g. n=3 for the -882 T/C variant, with SD´s of 0.4 and 3.4 between the groups). This is a recipe for outliers to have undue influence. A non-parametric test i.e. Wilcoxon rank sum would be more appropriate for the calculation of P values for continuous variables.

Reply: As pointed out by the reviewer, the use of an ANCOVA model can be misleading when the data are skewed. Hence, we have performed non-parametric tests (2 independent samples, Mann-Whitney Test / Wilcoxon rank sum, age=covariate) for the analyses on the -882T/C variant, and with these tests the difference turned out to be non-significant (p=0.059).
The same statistical procedure performed on the Pro12Ala and 2604T>C variants (non-parametric test, K independent samples, Kruskal-Wallis Test, age & BMI=covariates) also gave non-significant outcomes, p=0.100 and p=0.215 respectively.
However, one of the problems using non-parametric tests is that any effect of possible covariates (i.e. age and BMI) cannot easily be adjusted for, hence significant effects (which may be true) are likely to disappear when using the non-parametric tests.
However, we agree that the non-parametric test may be the appropriate choice also in our sample when data are skewed, and we have thus removed these non-significant findings from the "Results section", page 7 line 100.

Q3 (p9):
It is a little confusing why the -1881 was not studied further due to tight linkage with -1505, then it turned out that the -816 and -2604 were also in complete linkage with -1505 but they were still genotyped in everyone. Why was this not apparent from the initial scanning as it was for the -1881? There is nothing wrong with genotyping these variants in the larger set it is just not apparent why this was done for some but not all of these linked variants.

Reply: We found that the variants -816C>T, -1505G>A, -1881C>T and -2604T>C, respectively, were in complete linkage disequilibrium (LD). However, only the -2604T>C variant was studied further. Hence, the correct description should have been:
"Also, the initial analyses showed that the variant -1881C>T was tightly coupled to the -2604T>C (NOT -1505G>A) variant and was thus also not studied further (Table 1). [Insert Table 1]". The manuscript is revised accordingly, page 6 line 89.

Q4 (Table):
Presumably all the numbers in the Table are n´s. Label them as such, and include the % in each row for each genotype to enable easy comparison of the genotype distributions.

Reply: The numbers in the table are n´s. In the revised manuscript they are labelled as such, and we have included the % in each row for each genotype as suggested by the reviewer.

Reviewer 2

We thank the reviewer for the most valuable comments which have helped us to improve and clarify our manuscript.

Q1. It was found that the -882T>C polymorphism was associated with BMI in the obese but not in the lean cohort (top of page 10). It seems reasonable to find the association only within the obese group (top of page 10). However, the number of heterozygotes for this polymorphism is very small, making it inadequate for association studies. The -792 and Pro12Ala would have been a better indicator.

Reply: We agree that the sample size for the test of -882T>C variant with respect to phenotypic variable is quite small. Hence, in our discussion and final conclusion we have deliberately not made any final conclusions on these findings. However, due to the comments raised by reviewer 1, we have re-analyzed the data using non-parametric tests, which may be a more correct statistical method for this purpose. As these analyses actually turned out with non-significant p-values, we have revised the manuscript accordingly, i.e. concluding that all genotype-phenotype analyses did not suggest any genotype effects of either variant. Please see page 7 line 102.

Q2. Table 2. It appears that the sample size was not sufficient to detect linkage desequillibrium between a number of marker pairs. One would conclude that the -882, -2604 and the Pro12Ala are in very strong disequllibrium. Therefore, the Pro12Ala should also have been associated with obesity in the obese subgroup.

Reply: We report that the -882T>C variant is not in linkage disequilibrium with any of the other identified variants. However, the Pro12Ala variant is in strong linkage disequilibrium with the -2604T>C variant (R^2=0.93). In our initial ANCOVA analyses, this is also in accordance with our reporting that both the Pro12Ala variant and the -2604T>C variant are associated with lean tissue mass. However, as described in Q1, after using the non-parametric tests these associations were not statistically significant.

Q3. A previous study (Meirhaeghe, A et al. (1999) Int. J. Obesity 24:195-199) found an association of the Pro12Ala polymorphism with obesity in a much larger French cohort. Perhaps discrepancies between studies are due primarily to sample size differences. The results in this paper should be discussed. In addition, there are a number of publications on this topic that have not been mentioned in this manuscript. I would suggest putting all the positive and negative findings on associations of the Pro12Ala polymorphism with obesity in a table with the number of subjects, gender and age of the subjects.

Reply: Most interestingly, the study by Meirhaeghe A et al. found a significant association between obesity and the Pro12Ala polymorphism in a larger French cohort (n= 839) and in a recent meta-analysis by Masud S et al. J Med Genet 2003;40:773-780 it was also concluded that Ala12 homozygotes had significantly higher BMI than heterozygotes and Pro12homozygotes. Both studies are relevant to consider in the context of the present study, and both studies are included in this manuscript. As suggested by the reviewer, the discrepancies between our findings and the study by Meirhaeghe and the meta-analysis could be due to sample size. However, it should be noted that the 2 largest studies to date (n=1600, Memisoglu A et al. Pharmacogenetics 2002;12:597-603 & n=2245, Frederiksen L et al. J.Clin.Endocrinol.Metab 2002;87:3989-92) did not report any evidence that the Pro12Ala variant was associated with obesity, but also that the literature on this issue is generally inconsistent. With this in mind, the aim of the present study was to determine if the inconsistent results of previous studies could be explained by other gene variants in the PPARgamma promoter region, whether these variants were in linkage disequilibrium with the Pro12Ala variant or not.

Finally, including a list of all findings on Pro12Ala and obesity (and type 2 diabetes) within the manuscript limitations for publication in "Brief Genetic Analyses" section of Obesity Research would not be feasible or acceptable according to the Journal policy. However, such lists can be found in the meta-analysis by Masud et al., although more studies on the topic have been published since that analysis was reported.

Q4. Perhaps it would be good to determine the frequency of haplotypes generated by -792 promotor variants, the Pro12Ala and the +1431 polymorphisms and perform associations with haplotypes instead of single polymorphisms.

Reply: Haplotype analyses of haplotypes generated by the variants -792A>G, Pro12Ala and +1431C>T may be substantiated due to their relative frequency and/or theoretical speculations on their individual and combined influence on PPARgamma transcriptional activity. However, due to the limited sample size this may not be appropriate. Combinations of these 3 gene variants can theoretically give rise to a total of 3*3*3=27 different haplotypes. However in our sample, the actual number is 15 different haplotypes, of which n=1 for three of the haplotypes. Thus, though the idea is very tempting, it is not likely to be successful to perform such analyses with the available sample size.

Feb 25, 2005

To Associate Editor
Lee M. Kaplan, MD, PhD,
Obesity Research

Re: Revised version of MS# 04-113
Identification of novel variants in the putative PPARγ2 promoter and relationships with obesity in Danish men

Dear Dr. Kaplan,
We are most grateful for the opportunity to provide you with a revised version of the manuscript. Below, we have addressed the remaining points raised by reviewer 2, and changes in the manuscript are marked with bold type.
We hope that our reply appropriately addresses the points raised, and that the manuscript will be acceptable for publication. Due to the previously very long reviewer processing time we sincerely thank you that you will expedite and conclude the editorial handling of our paper.

Points raised by reviewer 2
I still have problems with linkage disequilibrium between markers. It appears to me that all the promoter polymorphisms should be in LD with each other and with the Pro12Ala and that all of these lie within a single LD block. The observed lack of LD is most likely due to low power of detecting LD, particularly if they are in negative LD. The conclusion from this work is that the promoter polymorphisms do not explain the reported discrepancies in the association of the Pro12Ala polymorphism with obesity and type 2 diabetes. This should be clearly stated in the abstract and discussion. I believe that the most likely reasons for such discrepancies are sample size, ethnicity and population stratification.

Reply:
We have detected LD between pairs of variants using R^2 and have found complete LD between 4 novel variants and the Pro12Ala variant, which all had an allele frequency of around 15 %. We agree with the reviewer that we have limited power to detect LD due to our sample size, but the other determined variants in the promoter all have different allele frequencies and might therefore not be in any significant LD with each other. However, this does not rule out that these variants together form haplotypes with limited variance in the linkage block surrounding the PPARG locus.

We acknowledge that the discrepancies between the present study and previous findings may be due to differences in sample size, ethnicity and population stratification. This is now stated in the discussion (page 8, line 124) part of the manuscript.

Finally, we agree that our findings infer that the overall conclusion is that "the identified novel variants in the *PPARγ2* promoter region do not explain the reported discrepancies in the association of previous identified variants with obesity and type 2 diabetes". Hence, this has been inserted into both the abstract (page 3, line 20) and into the conclusion part (page 8, line 136) of the manuscript.

June 26, 2005

Point to point reply:

EDITORIAL COMMENTS:

The file formats submitted were appropriate for review purposes, but cannot be used for publication purposes. Before resubmitting your manuscript online, please either transfer each figure into a separate PowerPoint file or save each figure as a separate TIFF file. The title page, body, references, and tables can be submitted in either Microsoft Word or Word Perfect format. Please contact Sharon or Zann at the Editorial Office if you need help with this.

Answer: Figure 1 is provided as a PowerPoint file, and the title page, body, references, and tables are submitted as a Microsoft Word file.

We have not received a signed Authors Agreement form for your paper. Please download one at http://www.ajcn.org/misc/agree.pdf, have all authors sign it, and return it by fax to 1-530-752-8371.
Manuscripts can not be accepted for publication in the AJCN without this form signed by all authors.
All manuscripts submitted to the AJCN must have line numbers in the margin throughout the abstract and text. Our office staff added line numbers to the first version of your manuscript. The revised manuscript must be submitted with line numbers.

Answer: A signed Authors Agreement form will be provided by fax within the next days.
The current revised manuscript has been provided line numbers throughout the abstract and text.

INTRODUCTORY NOTES:

The authors have just recently been informed that the dietary supplement used in the study was CLA given as triacylglycerol (CLA-TG) and not as free fatty acids (CLA-FFA).
We apologize sincerely for this, and we have now incorporated this new information to the manuscript where appropriate, i.e. in the introduction and methods section.

REVIEWER 3:

1. Trade names should not be used within the text; generic descriptors should be used instead. Such items should be identified once in the paper, in the Methods section in parenthesis. The name and location of the manufacturer should also appear within the parenthesis.

Reply: We agree, hence as suggested by the reviewer, the trade name is deleted from the text and identified in the Methods section instead, as well as the name and location of the manufacturer.

2. The abstract should be written in complete sentences and limited to 250 words. The Introduction should be limited to 1.5 pages (~450 words) and the Discussion to 4 pages (~1200 words).

Answer: The current manuscript is revised, so that it fulfils these requirements. In this regard, please note that parts of the introduction has been moved from the introduction part to the discussion part of the manuscript.

3. Please use US spelling. Center, randomization, tumor etc

Answer: We have revised the manuscript using US spelling.

4. Line 87. Please replace "female subjects" with "women".

Answer: We have replaced "female subjects" with "women".

5. Line 161-162 should be moved to the beginning of the Methods section.

Answer: We agree, and have moved line 161-162 to the beginning of the Methods section.

6. Line 170. The rationale for using the last-observation-carried-forward protocol is that this will make it MORE difficult to find a significant effect. When dealing with weight loss/weight regain data it will make it LESS difficult to find a significant effect. (the drop outs are more likely to be the individuals who could not maintain the weight loss and therefore the mean weight gain data are probably too low). Therefore this analysis should either be deleted from the paper or this perspective should be clearly stated when the results are reported.

Answer: The motivation of ITT is not to make findings more difficult, but is preferred as a method of analysis because a study should have the robustness to resemble real life. We admit that our use of ITT & last-observation-carried-forward analyses are not well described, and that it actually differs slightly from standard use (usually all subjects who sign the informed consent and engages in the study).
In our study we define our ITT population and we perform our last-observation-carried-forward analyses on the subjects who has completed 26 week treatment (i.e. body weight and DXA-data are available at week 26). Therefore, the number of subjects used for the analyses of 1 year treatment effects are 83 (The sum of the 77 subjects who completed the 1 year study + 6 subjects who were withdrawn or lost during the last 26 weeks treatment). In this respect, we note that the distribution of drop outs during the last 26 weeks treatment seems not heavily influenced by treatment (CLA=2, Placebo=4).
We have described this more clearly in the revised manuscript, and the figure 1 has been revised accordingly.

7. Page 8, line 173. It is not obvious in figure 1 why you only had an ITT population of 83 rather than 101. (Also lines 185-187).

Answer: As described to point 6 above, the use of LOCF and ITT analyses have been described more clearly, and the figure 1 has been revised accordingly, also explaining how the ITT population consists of 83 persons.

8. The statistical methods used in this paper tell us that two means are not significantly different, not that they are similar. There is an important, but subtle difference. Additional tests are needed to determine that two means are the same. Please use phrases such as "not different" rather than "similar", "same", "comparable" etc.

Answer: We agree that this is a subtle, but important difference, and we have revised the manuscript accordingly.

9. Line 201. The word baseline is used twice in this sentence. Once is enough.

Answer: Is revised.

10. Line 207. Please don't use the abbreviation NS in the text. Spell it out.

Answer: Is revised.

11. Line 223. A pregnancy might be an adverse event as far as the researchers are concerned, but hopefully it was not an adverse event for the woman. Could this sentence be modified slightly?

Answer: We agree, and have revised accordingly.

12. Leukocyte concentrations should be included in the table.

Answer: We agree, and the data for the Leukocytes have been inserted in the table.

13. Figures 2 and 3 are not called out in the paper. Data should be presented only once in the paper, not in both tables and figures, and therefore they should be deleted.

Answer: We believe that the presentation of the body weight change throughout the study is important to the reader, and thus we would like to keep it in. Figure 2 is now called out in the results section.
We agree, that figure 3 is not necessary as the data are also presented in table 1, and figure 3 is thus deleted.

14. Table 1. Footnote 1 should be modified to read: P value from unpaired t test except for gender where Fisher's exact test was used. The footnote should then be placed on each of the two column headings "P-value between groups". Footnote 3 should be deleted and the other footnote numbers changed.

Answer: As the table 3 has been deleted, and most of the table 2 has been deleted/inserted into table 1, the foodnotes to table 1 has been changed accordingly.

15. Much of the after LCD data are reported in both tables 1 and 2. This should be avoided.

Answer: The most relevant LCD data from table 2 are inserted into table 1, and thus table 2 has been deleted.

16. Table 2 should have been analyzed with a 2- factor repeated measures ANOVA with interaction which includes the 25 week data. Because there were significant differences at 0 week, it would be appropriate to also control for the 0 week data in the analysis.

Answer: For analyses of changes (Δ 52–0) in body weight, FM, FFM and FAT%, covariance analysis (ANCOVA) was performed using the week 0 value and the body weight change (week 0 minus week -8), treatment center and gender as covariates.
All though a repeated analysis could be used, we believe that this would give essentially the same results.
One other concern is the fact that the statistics described in the protocol, and which was submitted for review and approved by the local ethical committee, only included ANCOVA analyses and not repeated measures ANOVA.
It is recommended by the medical authorities (including the Danish Medicines Agency) that all measures are taken to ensure transparency in the execution of medicinal clinical trials, including statistical analyses and reporting – and therefore they recommend that the results are reported in the same way as initially described in the approved protocol.
All though we realize, that CLA is not considered a medical drug (but could be considered so in the future?), and all though we agree that the statistical descriptions given in the protocol should not prevent the use of other statistical analyses, if arguments are in favour of this, overall we believe that a repeated analysis would not give any substantial extra value to the manuscript.
Other changes (waist, hip & energy intake) was analysed using unpaired t-test.

17. Table 3 should be deleted. The first three columns should be summarized in footnote form to table 1 and the right hand 3 columns should be deleted.

Answer: The table 3 has been deleted, and the most important information from the first three columns are summarized in a footnote to table 1.

18. Figure legends should include both the title of the figure and any explanatory information – number of subjects, identification of error bars, results of statistical analysis etc.

Answer: The figure legends and explanatory information has been revised, so that it includes both title, number of subjects, identification of error bars, results of statistical analysis etc.

REVIEWER 1:

Comments:
This study evaluated the effects of 3.4 g/d of mixed CLA (40% each of cis-9,trans-11 and trans-10,cis-12 as FFA) on body weight and body composition after major weight loss in obese men and women. The study design involved a 10-wk weight loss diet followed by randomization and 12 months of monitoring while taking either CLA or placebo. Despite the double blind randomization procedure and similar drop-out rates, the two study groups upon completion of the protocol were found to have been mismatched for initial body weight and BMI. Correcting for this inequality at baseline, however, there was no effect of the 12-month CLA supplementation of the rate of weight regain compared to placebo. Given similar frequencies of reported adverse events between the two groups and no significant effects on glucose and a panel of hormonal variables, the authors conclude that this formulation of CLA is as safe as placebo (olive oil).

This study appears to have been well designed and carefully performed. The weight disparity between groups is an unfortunate consequence of blinded randomization without stratification for weight, but even this procedure cannot prevent disparities due to differential weights of dropouts. The authors are to be complemented for retaining 77 of 101 randomized subjects thru a 12 month protocol.

The lack of a significant effect of this CLA formulation of body weight and body composition is an important observation. The lack of an overt treatment effect on insulin resistance is also a significant observation.

This reviewer's primary concern with the manuscript is the dismissal of the change in leukocyte concentration with CLA as inconsequential (lines 282-3). The significant increase in total leukocytes across the 12 months of CLA supplementation was 0.77 vs 0.19 x10ninth for placebo (P=0.03). Since the reports of Friedman (NEJM 290:1275-8, 1974) and Kannell et al (JAMA 267:1253-56, 1992), multiple additional studies have corroborated total WBC as a robust predictor of coronary risk within the "normal range" (see also Brown DW et al, J Clin Epidemiol 54:316-22, 2001). This net increase in total WBC of 0.6 in the present study predicts an increase in coronary risk on the order of 20%, and is thus should not be dismissed "minor". In discussing the importance of this observation, it would be very helpful if the authors could include the results of serum hs-CRP across the 12 month supplementation period in these subjects as well. Particularly if there was an elevation in CRP with CLA in this protocol, the authors should consider revising their assessment of the safety of CLA as equivalent to that of olive oil.

Answer: We agree that this information could be given more emphasis. Note that the numbers have been recalculated (now CLA group ($0.81 \pm 1.21\ 10^9$/L) than placebo ($0.19 \pm 1.14\ 10^9$/L), p=0.033) which is essentially unchanged.
Accordingly, we have changed this sentence into:

"Only leukocyte concentrations seemed increased by 1 year CLA treatment. Similar observations have been reported in previous long-term studies(7;10;18). All though the actual increase in leucocytes is generally small, this increase may be of some concern as previous studies have indicated that leukocytes are an important indicator of inflammation and has also been identified as an predictor of CHD mortality(19). However, as the values are within reference values [3.0-10.0 10^9/L], and as CLA in most cases are used for a limited time period, the clinical relevance of this finding is still unclear. Also, a recent 12 week study in humans actually suggested that CLA may have beneficial effects on immune function(25)."
Also, we have changed the abstract and conclusion slightly to mention this observation.
We note that the changes in platelets (thrombocytes) did not change significantly different between groups (p~0.6).
We agree that data on hs-CRP could add important information to this issue, but unfortunately we don´t have any data on hs-CRP.

REVIEWER 2:

Comments:
There are now several published studies that have reported the effects of CLA on body weight and body fat in humans. Only one research group in Norway has reported in various publications that CLA may reduce body weight and fat, but the effects were relatively small and not always significant and consistent. All the other studies published by other research groups did not find any significant effect. The present study confirms this observation. The present manuscripts provides interesting information. However, there are several issues that need attention.

1. Line 39: Reviews. Only one review is quoted.

Answer: We have changed accordingly, now only one review is quoted.

2. Line 44 and line 102 : Other fatty acid isomers and others. Commercial CLA preparations are made by isomerisation of linoleic acid and contain usually 40% of the 9,11 and 40% of the 10, 12 CLA isomer. The remaining 20% are usually other fatty acids and and only a small amount of other CLA isomers. The expression "fatty acids isomers" and "others" is not very clear and specific. E.g. "others" could mean other fatty acids or other CLA isomers.

Answer: We agree, and the description has been changed accordingly into "the remaining 20% are usually other fatty acids; approx. 1-4 % other types of conjugated fatty acids and 15-19% other non-conjugated fatty acids".

3. Line 47: A reference should be given that describes the CLA intake.

Answer: We agree and a reference has been given.

4. Line 52: CLA may be useful in treating diabetes. A large number of studies in mice have clearly shown that CLA induces diabetes and a similar trend has been found in pigs (Stangl et al.), and hamsters (Bouthegard et al.). Also in a previous publication (JLR), the authors have described that CLA has severe side effects like diabetes and hyperinsulinemia. Only in diabetic rats, CLA has been shown to improve diabetes (Henriksen et al, and some other publications in obese and diabetic rats). In addition. CLA induces in mice a fatty and enlarged liver and similar results have been reported in hamster studies (Dedeckere et al). Further, various studies by the group of Vessby et al. suggest that CLA may lower insulin sensitivity. Only one study in humans have reported an improved insulin sensitivity (Eyjolfson et al. Med. Sci. Sports Exercise 36: 814, 2004) Thus, most of these studies suggests that CLA rather induces diabetes than that CLA improves diabetes.

Answer: In the JLR review, we have not specifically provided evidence that CLA is able to induce clinical diabetes in humans! However, we agree that CLA´s effect on insulin sensitivity & type-2 diabetes is highly controversial and we have adjusted the description in the present manuscript accordingly, into: "Hence, CLA has been suggested to be useful in treating diabetes by controlling body fat and weight gain(7), but other recent studies in humans have indicated that CLA may actually have negative effects on insulin sensitivity(8)".

5. Line 67: The only two publications: A third study has now been published in J. Nutr. April issue, and one of the authors of the present manuscript is also author of this publication. In this manuscript, CLA appears to be able to prevent weight gain and the results of this publication should be discussed. The discrepancy between between the present study and the publication in J. Nutr. shows again that the results found in the Norwegian studies could not be reproduced by other research groups. The reason for that is not clear.

Answer: The current study design is novel, in that it studies the efficacy of CLA to decrease weight gain (i.e. AFTER an initial weight loss). We are only aware of 2 previous studies (Kamphuis et al, 2004 & Whigham LD et al. 2004) using a similar design. The mentioned study (Gaullier J-M, 2005, J.Nutr) is not a weight regain study, Therefore the design is not directly comparable. However, we have now made a reference to this study in the discussion section.

6. Fig 1. and the number of subjects. Figure 1 does not appear to be clear. In the Tables, the CLA group comprises 40 subjects and the control group comprises 43 subjects. None of these numbers can be found in Fig. 1. However, scrutinizing the text learns that the 40 subjects in the CLA and the 43 subjects in the control group completed the 25 week period, whereas only 38 subjects in the CLA group and 39 subjects in the control group completed the whole 52 week period. This should be clearly stated in Fig. 1. Figure 1 could also be simplified. For example:

135 subjects
?
122 involved in Low Calorie Diet
?
101 subjects randomized
?
51 in CLA 50 in placebo
? ?
40 in CLA 43 in placebo (completed 25 weeks)
? ?
38 in CLA 39 in placebo (completed 52 weeks)

Also the sizes of the corresponding boxes should be the same. Also the abbreviation AE should be clarifed in a foot note to this figure.

Answer: Reviewer 3 did also find the figure 1 unclear, and we agree that it is not very well described.
We have revised the figure 1 including the abbreviations and footnotes.

7. Line 170: Last observation carried forward. Does this mean that the 25 week values of the subjects that did only complete the 25 week period were also used for the 52 week period? This should be more clearly mentioned for the reader who is not familiar with this concept. This should also be mentioned in the footnotes of the Tables.

Answer: Yes, the 25 week values of the subjects that did only complete the 25 week period were also used for the 52 week period! We have described this more clearly in the methods as "Also LOCF was used for the subjects who completed the 26 weeks treatment, but did not complete the 1 year period, for DXA and weight variables",
This has also been added as a footnote to table 1.

8. Line 14, Line 115, Table 2. The authors use sometimes kJ and sometimes calories. This is very confusing and either calories or kJ should be used. kJ could be used (metric system) and within brackets calories and indicate that 1 kcal = 4.18 kJ.

Answer: We agree, and have revised accordingly, so that kJ are used throughout the manuscript.

9. Line 109: Power analysis to estimate the number of subjects needed.
This is usually difficult, since the expected standard deviation has to be known, although a standard deviation can be estimated from other, similar studies.

a) In the present study, it is calculated that if there is a difference in body weight regain of 2.2 kg between the groups, how many subjects are needed to pick up this effect.
b) What is the reason for using 2.2 kg as a criterium (because of the results of the studies of Gaullier)?
c) What was the estimated SD that has been used for these calculations?
d) Table 2 shows that the SD of the difference in body weight between week 0 and week 52 is 4.99. If one wants to pick up a difference of 2.2 kg and the SD of these differences is 4.99, then the number of subjects needed is 132 per group (p value <0.05 and power of 90%, unpaired t-test of the differences between week 0 and week 52, can be calculated with statistics computer programs or with power analysis programs found on the internet).
e) Since the calculations of the number of subjects needed is difficult because of the unknown SD, I would suggest to leave out this issue in the manuscript, unless the procedure is being more clarified.
f) Further, what means (line 110) "efficiency size estimated"? Where does the number 1.7 come from? This is not clear to the reviewer.

Answer:
a) We agree that accurate and meaningful power analyses are very difficult to perform, particularly when using novel study designs. However, many journals now encourage that descriptions of power analyses are reported in the manuscript, all though they may not always be meaningful. In the present study, the power analysis was based on changes in fat mass regain (not body weight regain).
b+c) The 1.7 effect size was based on a previous study of Blankson et al. J.Nutr. 2000, where a 3.4 g CLA/day over (only) 12 weeks induced a fat loss of 1.7 ± 1.9 kg (mean ± SD). The SD at 2.2 kg was assumed to be detectable differences in fat mass using DXA-methodology, based on prior experience on studies performed at RVAU. I.e. we had prior experience of within-group SD~2.2 kg fat mass using DXA.

d) Retrospectively, we agree that the estimate of SD of 2.2 kg was lower than the observed ~5.0 kg.
e) As we would like to inform the reader about the reasoning about the sample size (and to comply with most journal guidelines), we have revised the description of the sample size calculation, and hope that this current description is clear to the reader: "Assuming a difference of 1.7 kg of fat mass between treatment groups based on prior studies using 3.4 g CLA/day vs. placebo(9) and an estimated SD of 2.2 kg (based on prior experience of within-group detectable differences using DXA-methodology) an estimated number of 37 subjects pr. group [*please note the change from 28 to 37 per group*] were required (using 90% statistical test power and 5 % significance level)".
Calculations performed via http://hedwig.mgh.harvard.edu/sample_size/quan_measur/para_quant.html
Also, in the discussion we now mention that "given the finding of a numerical difference of 0.6 kg fat mass between groups and a SD of ~5 kg, the study may have been inadequately powered".
f) See point b+c above.

10. Line 115: Why were the subjects fed a modest hypocaloric diet during the study? What was the rationale for that?

Answer: The study was performed in overweight and obese (28<BMI<35), who after an >8% weight loss should be given CLA or placebo. Of ethical reasons (the obese participants should have obvious benefits from participation) and in order to motivate the subjects to maintain the weight loss, they were instructed to follow a hypocaloric diet.

11. Line 161 and 162: This does not appear to belong to the ethics section.

Answer: We agree, and it is moved to the methods section.

12. Line 177: ITT The abbreviation is explained on the next page, but should be explained here, where it is being used for the first time. Maybe, such abbreviations should not be used at all, these are not very common abbreviations and these expression are only used once or twice in the text. The same is true for abbreviations such as AE.

Answer: The ITT has been changed to Intention-To-Treat, and the AE is now given a separate explanatory note in the figure 1.

13. Fig. 2 and 3. These figures are not mentioned in the text at all. Figure 3 can be deleted, since all these data are already given in the Tables.

Answer: Figure 3 is deleted (see comment to reviewer 3, point 13). We believe that figure 2 provides some important information and we want to keep it, but we have also now mentioned the figure 2 in the text.

14. Table 1, 2, and 3: There is a large overlap between these tables. For example, data that are given in Table 1, are given in Table 2 again. I believe that the tables 1 and 2 should be merged into one Table. The data in Table 3 do not seem to give any additional information. Table 2 indicates that there are no effects at all between the CLA and the Control group, thus the additional information that the percentage changes are also not different appears to be a kind of redundant.

Further, all the tables should have the same format and layout. This makes it easier for the reader to grasp quickly the results. For example, in Table 2, the CLA and Placebo groups are given in columns, but in Tables 1 and 3 in rows. The following layout could be used.

	Before LCD	Week 0	Week 25	Week 52
Weight				
CLA				
Placebo				
Fat Mass				
CLA				
Placebo				

This way, the results in each group can be easily followed in the time and the two groups can be compared easily at each time point. The same format should be used for all the Tables. Statistical differences between the CLA and the Placebo group could be indicated with superscripts.

Answer: We agree with the reviewer, and we have deleted table 3, and have merged table 2 into table 1. Likewise, the table 1 and table 2 (the previous table 4) has now been changed to the same format and layout.

15. Table 2. The DXA weight can be left out. The scale weight indicates that the regain is exactly the same in the two groups, and the DXA weight does not give any additional information. Further, the results in Table indicate that the regain is almost identical in the two groups and I believe that giving p values in the range of 0.5 – 0.7 is kind of redundant.

Further, for every parameter, the number of subjects is repeatedly given. The number of subjects could be given in a footnote and then also the age and the number of male and females could be given in this footnote. Why is the number of subjects for the energy intake data different from the other numbers? This should be clarified. The energy intake at week 0: Is this the intake that has been calculated, because at week 0, the subjects just finished the low calorie diet. It is not clear what this represents.

Answer: We agree, and have deleted the DXA weight data and the table 1 have been revised according to the suggestions; the number of subjects are given in a footnote, as well as the age and gender ratio.
The numbers of subjects for the energy intake reflects that not all subjects did report energy intake at all ime points. This is now clarified in the table 1.
By mistake, the baseline energy intake was described at week 0, which is incorrect. That has been changed into "Baseline", and thus there is no calculation of energy intake at week 0, where they have just finalized the LCD-period.

16. Statistics: Repeated measurements are done on the same subjects. It appears that the results of week 25 are not used for statistical analyses. Would it not be appropiate to do repeated measures analysis? This will, however, not affect the results, since it is very clear that there is no effect of CLA.

Answer:
All though we agree that a repeated analysis could be used, we believe that this would give essentially give the same results. Hence we have estimated that a repeated analysis would not give any substantial extra value to the manuscript.

17. Footnote 8 to Table 2: Why is an ANOVA test used? There are only two groups and an unpaired t-test could have been used. Reviewer statistically re-analyzed the data and the given p-value is actually the result of a simple t-test.

Answer: We agree, an unpaired t-test would be more appropriate. This is changed accordingly.

18. Footnote 1 to Table 3. Only two groups are compared, and an unpaired t-test could have been used.

Answer: We agree, however table 3 is now deleted.

19. Footnote 1 to Table 1. p-values between groups. In this case of gender, what is actually being tested?

Answer: The ratio distribution is being tested.

20. Footnote 1 to Table 3. Univariate analysis (ANOVA). Does this mean that a one way ANOVA is used? Why is here just an ANOVA used, whereas in Table 2 an ANCOVA is used?

Answer: We agree, that this seems inconsistent – and ANCOVA should have been indicated here for the analyses. However, the table 3 is now deleted.

21. Footnote 2 to Table 2. ANCOVA: In the text or in the footnotes, it should be mentioned that gender and week 0 value did not have any effects on the results. This is only mentioned in the Discussion section (Line 257). If the reviewer understands properly, the ANCOVA is being used to test whether gender, week 0 values, treatment center, and the effect of the low calorie diet has any effect on the results. The results of this test should be mentioned in the result section.

Answer: The reviewer is correct in that the ANCOVA is used to analyze the difference between treatment groups taking into account the week 0 values, the effect of the low calorie diet (change in body weight during LCD) as well as gender and treatment center.
For changes in body weight from baseline to week 52, the results indicated that both value 0 (p=0.046) and change in body weight during LCD (p=0.010) did influence change in body weight, whereas gender and center did not turn out statistically significant.
For changes in body fat mass from baseline to week 52, the results indicated that change in body weight during LCD (p=0.014) did influence change in body fat mass, whereas gender, center and initial body fat mass was not significant predictors of change.
However, for both analyses – as well as for analysis of FFM and FAT%, all parameters (i.e. initial body weight/fat mass/fat free mass/FAT%, and LCD-induced change in body weight, gender and center were kept in the ANCOVA analysis all though they did not necessarily affect the results directly.)

22. In general, the statistics are not always clear and should be described and executed carefully. This should be more clearly explained and kept as simple as possible. It should be explained why particular tests are being used and what the results of these test were.

Answer: We agree, that this has not been presented clearly, but we have now carefully revised the statistical descriptions throughout the paper.

23. Page 25: These are not Legends but Footnotes. The figure title and the number of subjects should be included in the legends.

Answer: That has been revised accordingly.

24. Line 260: Few mice studies. There are however, numerous studies in mice that show the deleterious effects of CLA, even when very small doses of 0.5% CLA (on weight basis) are given (See CLA website of the Wisconson University). These doses are comparable to the doses given to humans when expressed per kg metabolic weight or expressed as gram per 1000 kJ energy intake.

Answer: We agree, that there are indications that even quite small doses of the t10,c12 isomer has shown deleterious effects in some animal studies, and we have changed the description accordingly into: "However, some studies in mice have shown that particularly the t10,c12 isomer may induce lipoatrophy, hyperinsulinaemia and fatty liver (19). This may occur at dosing levels that are comparable to the doses used in human studies(20). However, due to species differences, it should be emphasized that one should always be cautious when comparing human and animal studies. "

25. Line 292: We conclude that. The present study does not show any side effects, but other studies indicate that in some cases these side effects that are seen in animal studies may also show up in humans. Thus, one has to be cautious, and it seems to still be questionable whether the use of CLA can be considered as safe.

Answer: We agree, and have modified the abstract and conclusion accordingly, emphasizing the observation of increased leukocyte number and with the subtle change that "…CLA dietary supplement *may* be as safe as olive oil".

26. Inspection of Table 2 and Figure 2 and 3 shows that during the first 25 weeks, only an increase in FFM has taken place and no increase in body fat. During the second 25 weeks (week 25 – 52), there was only an increase in body fat, and no increase (rather a small decrease) in FFM.

Answer: The likely explanation for this observation is that subjects are – at least to some extent – depleted for water after finishing the LCD-period (i.e. loss of water bound to glycogen depots etc.). The change in FFM from baseline to week 25 can therefore – to some extent – be explained by repletion of water in the glycogen depots as well as the slight weight gain (which usually also includes gain of FFM as well as FM).
In contrast, the changes in FFM from week 25 to week 52 will only be caused by weight gain (and not by glycogen water repletion).
Another reason could be time-dependent changes in physical activity, i.e. the subjects may be more physically active during the first 25 weeks (i.e. gaining more FFM), and they may be less likely to engage in the same amount of physical activity during the last ~26 weeks (i.e. losing FFM). This could also give an explanation for the observed results.

27. It would be nice that the reader would have an overview of all the human studies done this far. Besides authors previous review (JLR), some other reviews (such as Kelley, Lipids 38:377, 2003, Terpstra, AJCN 79:352, 2004) could also be given and also the latest studies that are not mentioned in these reviews, for example the studies of Malpuech et al. (Int J Obes12:591, 2004, Gaullier et al (J. Nutr. 135:778, 2005), Tricon et al. (AJCN 80:614,2004) Petrodou et al. (Lipids 38:805 2003) should also be cited. Also the Wisconsin University website should be cited. This is a very useful website and gives an oveview of all the CLA studies.

Answer: We believe that the review by Larsen et al. (JLR, 2003) covers most relevant studies in humans, and with the addition of the 1-year and 2-year studies of Gaullier et al. published in 2004 & 2005, the most important studies are now mentioned. The review by Kelley et al does not add important imformation, and the studies by Petridou, Malpuech & Trican are only short term (6-8 week supplementation) studies which are not directly comparable to the results obtained in the present study.
However, for the interested reader, the Wisconsin Website could be useful, and is therefore added into the manuscript in the introduction and the more recent review by Terpstra, AJCN 2004 is now also mentioned.

In conclusion, the manuscripts provides interesting information and confirms the results of most of the other CLA studies, that CLA has no or minimal effects on body weight and body fat. However, the results should be described and presented in a more clear fashion. Further, careful attention should be paid to the statistics.

26 September 2005

Point to point reply:

GENERAL COMMENTS:

The lines 91-93: "The randomization sequence was generated by Scandinavian Clinical Research (monitor firm) using a simple block randomization code without stratification"

have been changed into:

"The randomization sequence was generated by Scandinavian Clinical Research (Contract Research Organization) using a simple block randomization procedure without any stratifications".

REVIEWER 3:

Q1: Please start the Introduction on a new page.
A1: The Introduction is now started on a new page (page 3)

Q2: Trade names should not be used within the text; generic descriptors should be used instead. Such items should be identified once in the paper, in the Methods section in parenthesis. The name and location of the manufacturer should also appear within the parenthesis. (lines 83 and 87 for example)
A2: The trade name "Clarinol®" has been deleted from line ~252.
The name and location of the Nutrilett LCD product is provided at line ~83 (Collett Pharma, Lysaker, Norway).

Q3: Line 89. Please write as …39% c9,t11 CLA and 41% t10,c12 CLA. (add CLA).
A3: Lines 85-90 have been revised to increase clarification.

Q4: The authors state in their reply that they believe that a repeated analysis would give essentially the same results. This should be tested and the results of the repeated analysis included in the paper. If the interaction is significant, subgroup analysis should be done using the Tukey test across time within group, not paired t tests.
A4: We have performed repeated analyses of changes in fat mass, fat free mass, fat percentage and body weight. The values for each variable at the time points (week 0, 25 and 52) have been compared between treatment groups, using change in body weight from week 0 minus weight before LCD, center and gender as covariates. We did not identify any significant treatment effects (i.e. time*treatment effect) on any of these variables (fat mass, p=0.651; body weight, p=0.840; fat%, p=0.657 and fat free mass, p=0.323).

The statistical methods of the repeated analyses are inserted in the manuscript (line 170-173) and the results are stated in line 210 as: "Repeated analyses provided essentially the same results (p = 0.65 for changes in body fat mass)".

Q5. Generally: Each table and figure should stand alone without reference to the text. Each table and figure should include an indication of the variability of the data (SEM, SD etc.), the results of the statistical analysis, and the n. Please identify in table footnotes and figure legends the statistical test used to analyze the data
A5: The figure legends and footnotes have been changed accordingly (line 353-373).

Q6. Figure 2. The results of the statistical analysis should be reported in the figure legend.
A6: The results of the statistical analyses are reported in the figure legend.

REVIEWER 1:
Comments:
This manuscript has been significantly revised in response to the prior reviews. Unfortunately the authors have not added serum CRP data to further examine the issue of inflammation and safety raised in review. In addition, the authors' comments concerning the significant increase in total WBC seen in this study are worded in such a way as to minimize concern for this observation.

The potential effects of CLA on CRP aside, the repeated observation in a number of independent human studies of increased WBC with CLA administration makes this an important point, and one which should not be minimized. Given that increased WBC count within the reference range is a robust predictor of chronic coronary risk independent of dyslipidemia, the fact that it was not associated with serious adverse events within this one-year scope study can not be interpreted as implying safety in the longer term. The argument that changes in inflammatory markers have not yet been linked to coronary outcome has been addressed by the study by Ridker et al (NEJM 352:20-28, 2005), in which it was shown that CRP reductions by statin therapy reduce coronary events independent of the lipid-lowering effects of this drug class. Thus, within this context, it is incorrect to equate the safety of CLA to that of olive oil.

Specific comments:

Line 24. Remove the word "possible". Given the P-value of 0.026 (i.e., less than 0.05), there was a significant increase in the leukocyte count attributed to CLA administration.
Reply: We agree, and the word possible is now deleted.

Lines 28 and 310. "may be as safe as olive oil" represents speculation unsupported by the data. Olive oil has not been shown to increase WBC or CRP in multiple independent human studies, and increased WBC within the reference range has been repeatedly demonstrated to be associated with cardiovascular disease risk.
Reply: We agree, and have changed accordingly – into: "One year 3.4 g daily CLA supplementation does not prevent weight or fat mass regain in a healthy obese population."

Line 293. delete "seemed". Leukocyte concentrations were significantly increased.
Reply: "seemed" is changed into "was".

Line 294. Replace "All though' with "Although".
Reply: All though is replaced with although.

Lines 297-298. The sentence beginning with "However…" is gratuitous, and should be deleted. The rise in the leukocyte count within the reference range is associated with increased cardiovascular disease risk, and there are no data on how long the leukocyte count remains increased after CLA administration.
Reply: "However" is deleted.

Appendices:

Appendices A-G: Background information

Appendix A: Map of 20 kb region of the PPARγ locus harbouring the Pro12Ala variant

Appendix B: Map of PPARγ region illustrating variant linkage disequilibrium within the proximal 20 kb region of the Pro12Ala variant (*rs1801282*) / position 12.368.125 on chromosome 3.

Appendix C: Table with nomenclature of the most common fatty acids

Appendix D: Natural origin of rumenic acid (c9,t11 CLA): rumenal synthesis from linoleic acid, and endogenous occurrence from delta-9 desaturation of trans-vaccenic acid.

Appendix E: Cellular metabolism and bioavailability of the 2 main CLA isomers c9,t11 and t10,c12

Appendix F: Industrially and naturally generated 18:1 trans fats

Appendices G-L: Paper 1 to 4 and point-to-point replies for paper 1 and 2

Appendix G: Paper 1: Larsen, T. M., L. H. Larsen, S. K. Torekov, J. Ek, E. Black, S. Toubro, A. Astrup, T. I. Sorensen, T. Hansen, and O. Pedersen, 2005, "Novel Variants in the Putative Peroxisome Proliferator-activated Receptor γ Promoter and Relationships with Obesity in Men," Obes. Res. 13, 953-958".

Appendix H: Point-to-point replies for paper 1.

Appendix I: Paper 2: Larsen TM, Toubro A, Gudmundsen O, and Astrup A, 2005, "One year conjugated linoleic acid supplementation does not prevent weight or body fat regain.," Am J Clin Nutr. **Submitted**.

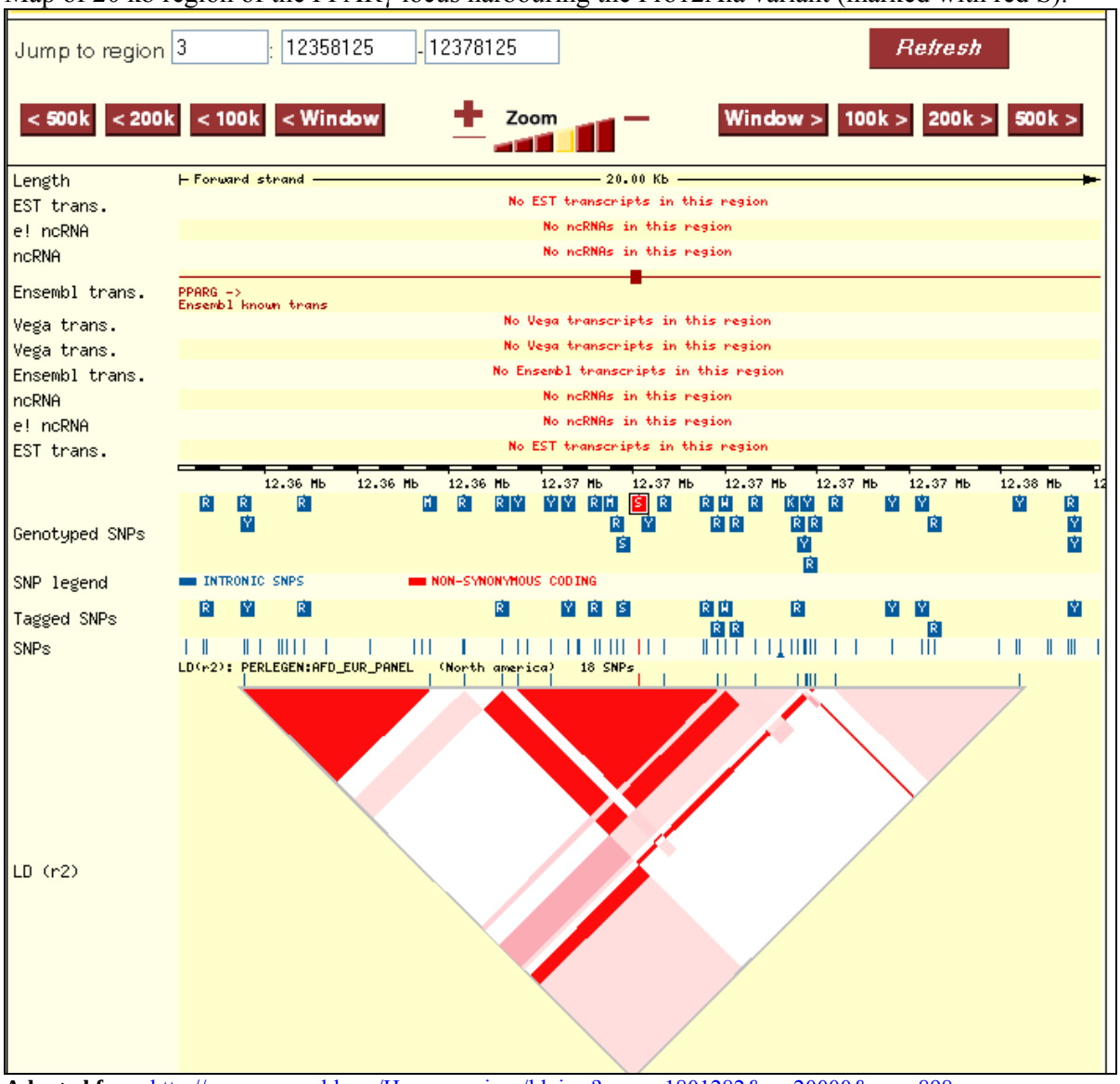
Appendix J: Point-to-point replies for paper 2.

Appendix K: Paper 3: Larsen, T. M., S. Toubro, and A. Astrup, 2003, "PPARgamma agonists in the treatment of type II diabetes: is increased fatness commensurate with long-term efficacy?," Int. J. Obes. Relat Metab Disord. 27, 147-161.

Appendix L: Paper 4: Larsen, T. M., S. Toubro, and A. Astrup, 2003, "Efficacy and safety of dietary supplements containing CLA for the treatment of obesity: evidence from animal and human studies," J. Lipid Res. 44, 2234-2241.

Appendix A:

Map of 20 kb region of the PPARγ locus harbouring the Pro12Ala variant (marked with red S).



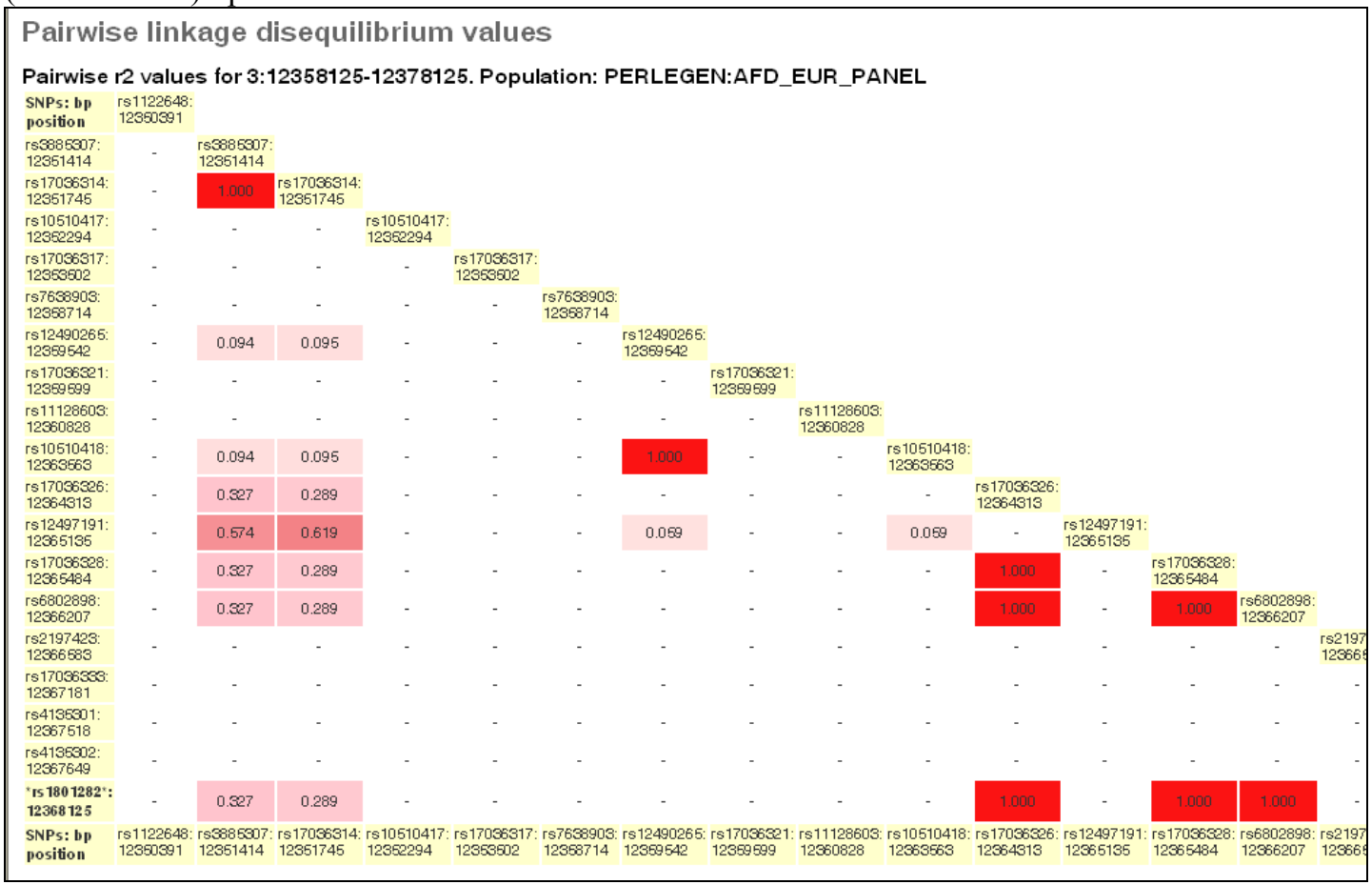
Adapted from http://www.ensembl.org/Homo_sapiens/ldview?snp=rs1801282&w=20000&pop=898
The map is based on 24 samples from the Coriell Cell Repository, primarily of European American descent.

Additional notes:
a) The PPARG gene can be found in the Ensembl Gene Report for ENSG00000132170
http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000132170;db=core

b) The PPARG protein can be found in the UniProtKB/Swiss-Prot accession number P37231
http://www.ebi.uniprot.org/entry/P37231

Appendix B:

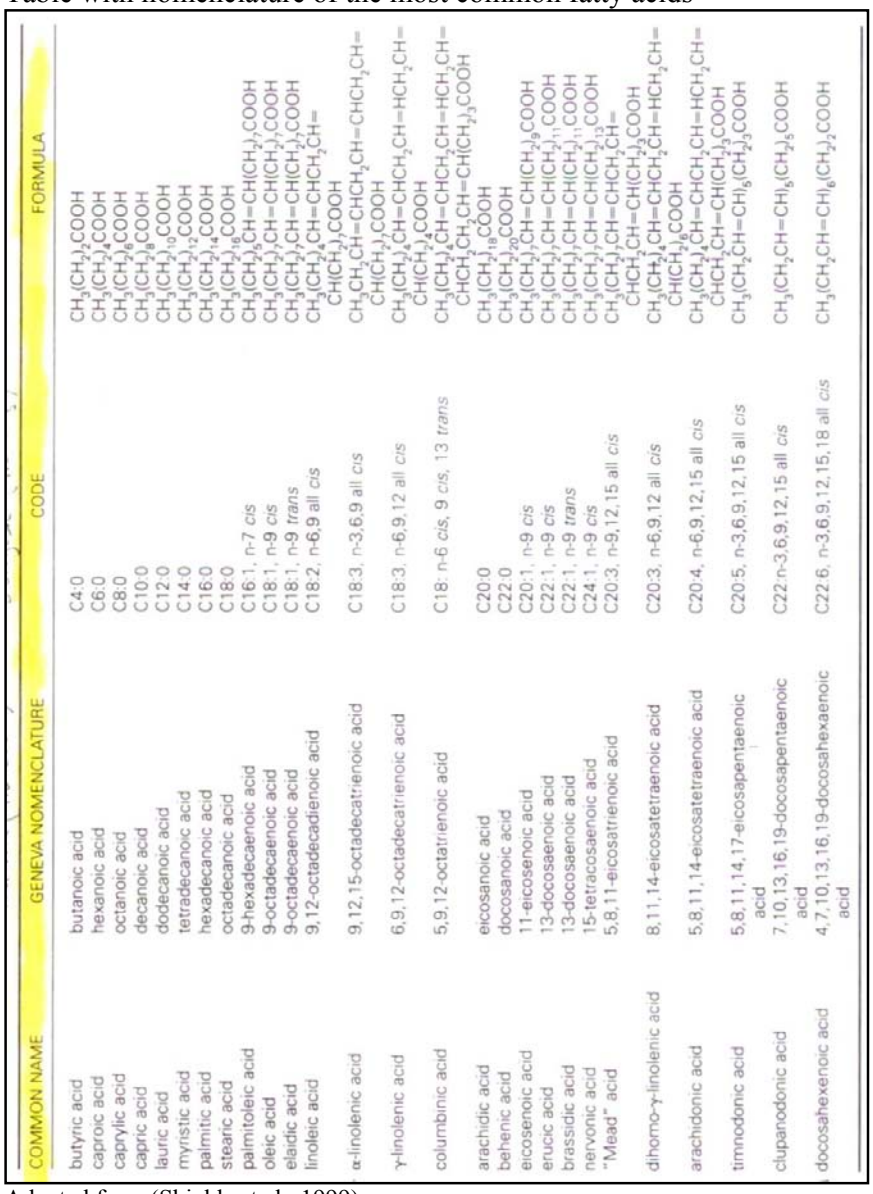
Map of PPARγ region illustrating variant linkage disequilibrium within the proximal 20 kb region of the Pro12Ala variant (*rs1801282*) / position 12.368.125 on chromosome 3.



Adapted from
http://www.ensembl.org/Homo_sapiens/ldtableview?_format=HTML&dump=ashtml&panel_image=on&w=20000&format_pdf=off&image_width=700&format_svg=off&format_postscript=off&snp=rs1801282&context=10000&pop=898&panel_options=on&submit=Dump

Appendix C:

Table with nomenclature of the most common fatty acids

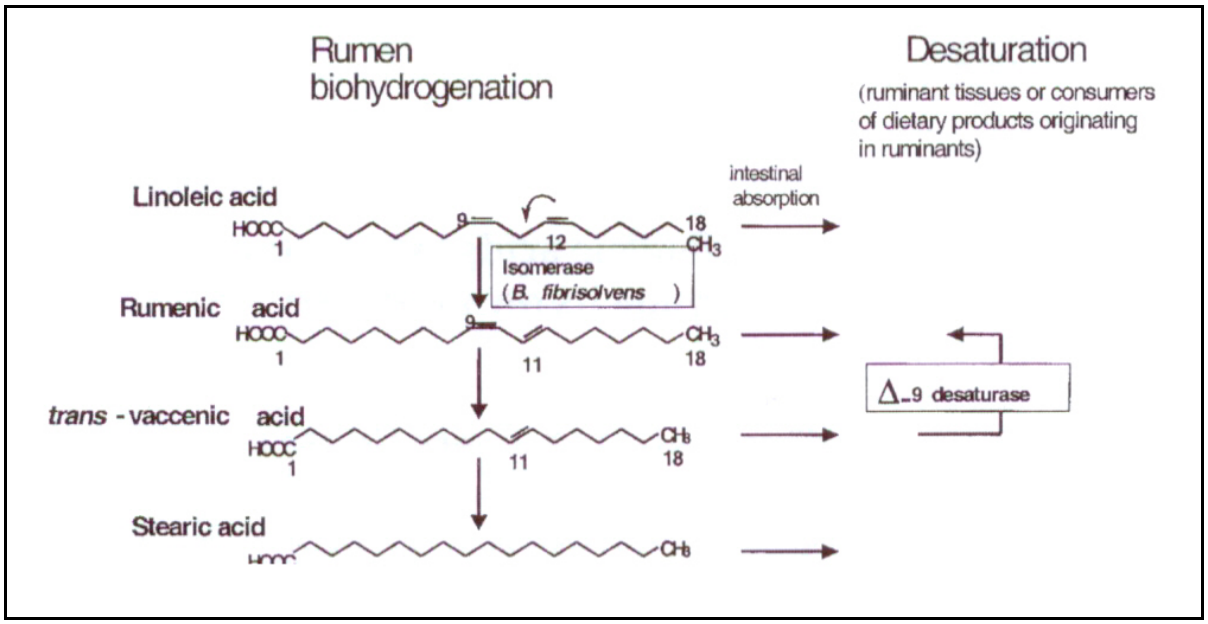


COMMON NAME	GENEVA NOMENCLATURE	CODE	FORMULA
butyric acid	butanoic acid	C4:0	$CH_3(CH_2)_2COOH$
caproic acid	hexanoic acid	C6:0	$CH_3(CH_2)_4COOH$
caprylic acid	octanoic acid	C8:0	$CH_3(CH_2)_6COOH$
capric acid	decanoic acid	C10:0	$CH_3(CH_2)_8COOH$
lauric acid	dodecanoic acid	C12:0	$CH_3(CH_2)_{10}COOH$
myristic acid	tetradecanoic acid	C14:0	$CH_3(CH_2)_{12}COOH$
palmitic acid	hexadecanoic acid	C16:0	$CH_3(CH_2)_{14}COOH$
stearic acid	octadecanoic acid	C18:0	$CH_3(CH_2)_{16}COOH$
palmitoleic acid	9-hexadecaenoic acid	C16:1, n-7 *cis*	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$
oleic acid	9-octadecaenoic acid	C18:1, n-9 *cis*	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$
elaidic acid	9-octadecaenoic acid	C18:1, n-9 *trans*	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$
linoleic acid	9,12-octadecadienoic acid	C18:2, n-6,9 all *cis*	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$
α-linolenic acid	9,12,15-octadecatrienoic acid	C18:3, n-3,6,9 all *cis*	$CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$
γ-linolenic acid	6,9,12-octadecatrienoic acid	C18:3, n-6,9,12 all *cis*	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}HCH_2CH{=}CH(CH_2)_4COOH$
columbinic acid	5,9,12-octatrienoic acid	C18: n-6 *cis*, 9 *cis*, 13 *trans*	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}HCH_2CH{=}CHCH_2CH{=}CH(CH_2)_3COOH$
arachidic acid	eicosanoic acid	C20:0	$CH_3(CH_2)_{18}COOH$
behenic acid	docosanoic acid	C22:0	$CH_3(CH_2)_{20}COOH$
eicosenoic acid	11-eicosenoic acid	C20:1, n-9 *cis*	$CH_3(CH_2)_7CH{=}CH(CH_2)_9COOH$
erucic acid	13-docosaenoic acid	C22:1, n-9 *cis*	$CH_3(CH_2)_7CH{=}CH(CH_2)_{11}COOH$
brassidic acid	13-docosaenoic acid	C22:1, n-9 *trans*	$CH_3(CH_2)_7CH{=}CH(CH_2)_{11}COOH$
nervonic acid	15-tetracosaenoic acid	C24:1, n-9 *cis*	$CH_3(CH_2)_7CH{=}CH(CH_2)_{13}COOH$
"Mead" acid	5,8,11-eicosatrienoic acid	C20:3, n-9,12,15 all *cis*	$CH_3(CH_2)_7CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_3COOH$
dihomo-γ-linolenic acid	8,11,14-eicosatetraenoic acid	C20:3, n-6,9,12 all *cis*	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}HCH_2CH{=}CH(CH_2)_6COOH$
arachidonic acid	5,8,11,14-eicosatetraenoic acid	C20:4, n-6,9,12,15 all *cis*	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}HCH_2CH{=}CHCH_2CH{=}CH(CH_2)_3COOH$
timnodonic acid	5,8,11,14,17-eicosapentaenoic acid	C20:5, n-3,6,9,12,15 all *cis*	$CH_3(CH_2CH{=}CH)_5(CH_2)_3COOH$
clupanodonic acid	7,10,13,16,19-docosapentaenoic acid	C22:n-3,6,9,12,15 all *cis*	$CH_3(CH_2CH{=}CH)_5(CH_2)_5COOH$
docosahexenoic acid	4,7,10,13,16,19-docosahexaenoic acid	C22:6, n-3,6,9,12,15,18 all *cis*	$CH_3(CH_2CH{=}CH)_6(CH_2)_2COOH$

Adapted from (Shields et al., 1999)

Appendix D:

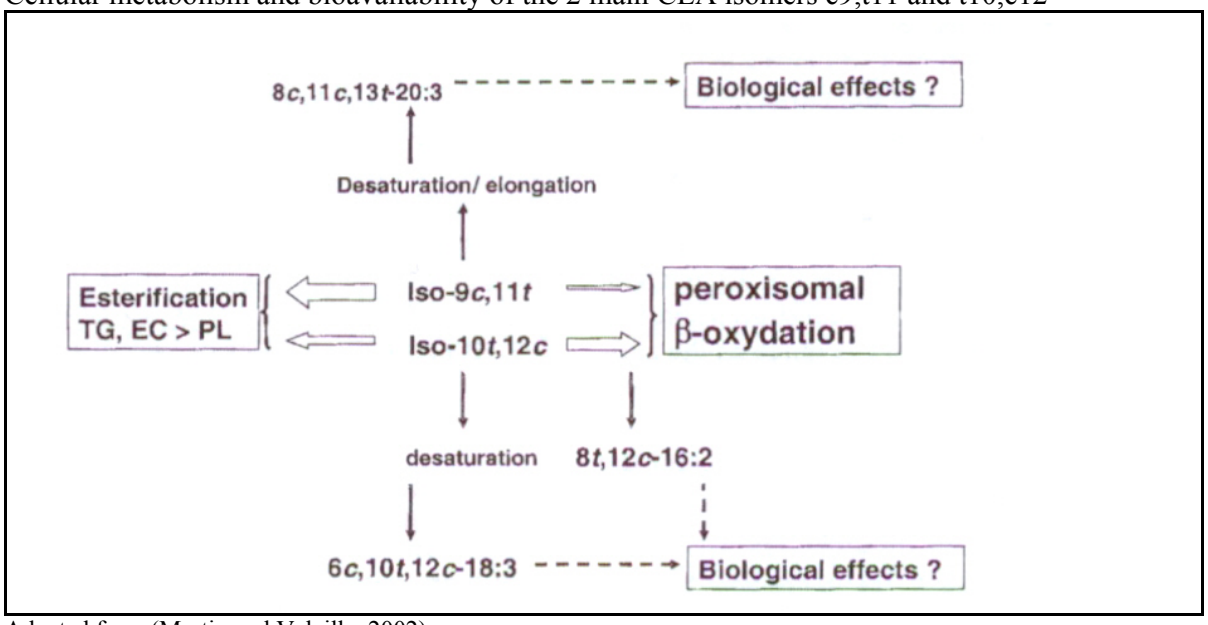
Natural origin of rumenic acid (c9,t11 CLA): rumenal synthesis from linoleic acid, and endogenous occurrence from delta-9 desaturation of trans-vaccenic acid.



Adapted from (Martin and Valeille, 2002)

Appendix E:

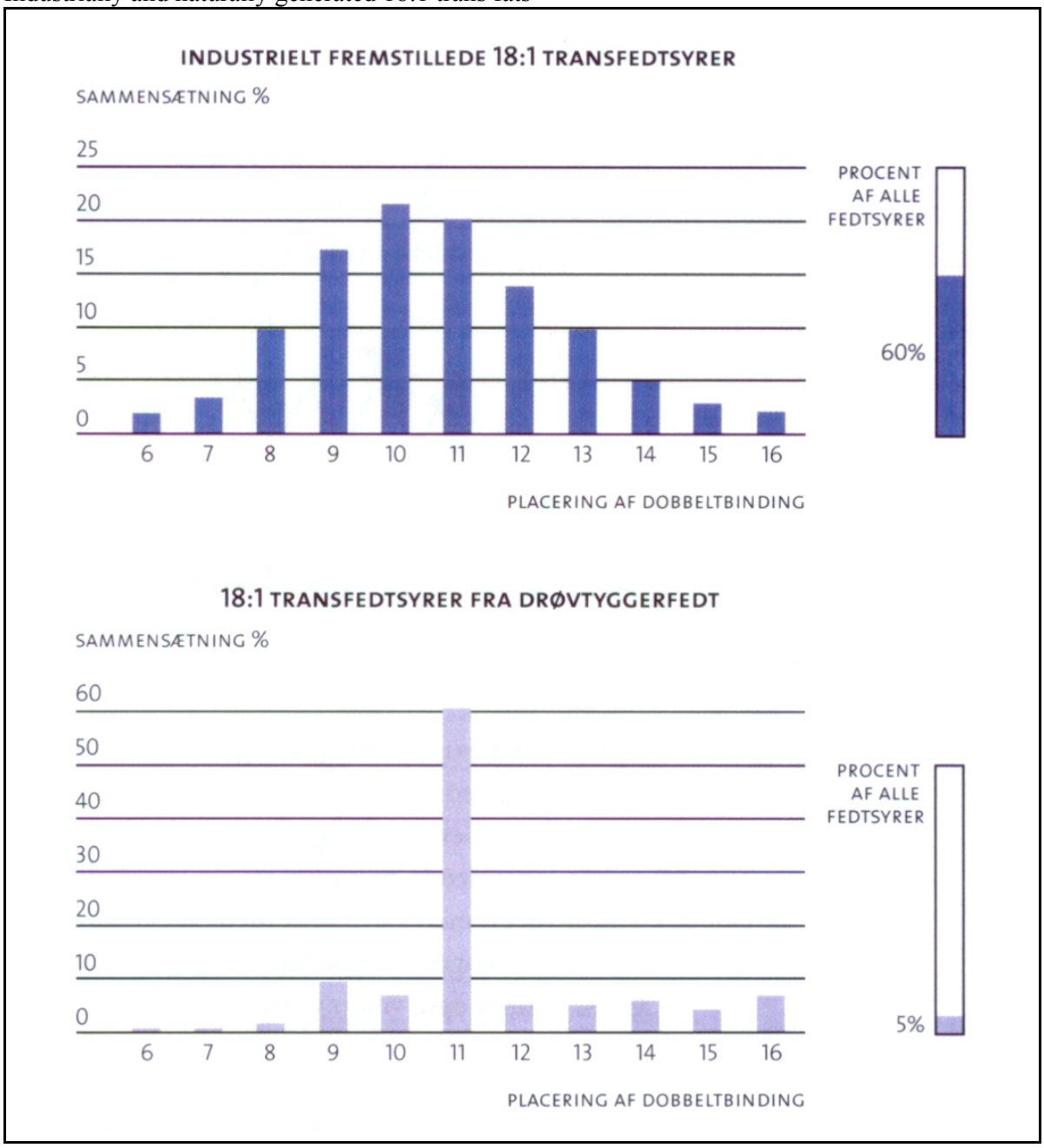
Cellular metabolism and bioavailability of the 2 main CLA isomers c9,t11 and t10,c12



Adapted from (Martin and Valeille, 2002)

Appendix F:

Industrially and naturally generated 18:1 trans fats



Upper graph: The composition of industrially generated 18:1 trans fats is highly heterogenic. The trans fats differ substantially regarding the position of the double bond (x-axis). The relative contribution from each of these fatty acids also differ substantially (y-axis).

Lower graph: The composition of 18:1 trans fats from ruminant fats. The trans fats from ruminant fats consists primarily of rumenic acid (c9,t11 CLA).

Adapted from (Steen Stender and Jørn Dyerberg, 2003)